10-07-05

AF/1617

IFW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Fred S. Lamb

Title: METHODS TO REDUCE THE SENSITIVITY OF ENDOTHELIALLY-COMPRISED VASCULAR SMOOTH MUSCLE

Docket No.: 17023.010US1
Filed: February 25, 2000
Examiner: Jennifer M. Kim
Serial No.: 09/512,926
Group Art Unit: 1617

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

We are transmitting herewith the following attached items (as indicated with an "X"):

X Return postcard.
X Appeal Brief including Claims Appendix, Evidence Appendix and Related Proceedings Appendix (114 pgs)

Please apply any charges or credits to Deposit Account No. 50-3503.

VIKSNINS HARRIS & PADYS PLLP
Customer Number 53137

By: Ann S. Viksnins
Ann S. Viksnins
Reg. No. 37,748

"Express Mail" mailing label number: EV703160221US
Date of Deposit: October 6, 2005
It is certified that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicates above and is addressed to Mail Stop Appeal Brief - Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Fred S. Lamb	Art Unit	: 1617
Serial No.	: 09/512,926	Examiner	: Jennifer M. Kim
Filed	: February 25, 2000	Docket	: 17023.010US1
Title	: METHODS TO REDUCE THE SENSITIVITY OF ENDOTHELIALLY-COMPROMISED VASCULAR SMOOTH MUSCLE		

APPEAL BRIEF

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The Final Office Action for this application was mailed November 30, 2004, and a Notice of Appeal was mailed April 14, 2005. An Appeal Brief was filed on April 22, 2005. However, as indicated in the Notification of Non-Compliant Appeal Brief mailed September 7, 2005, the April 22, 2005 Appeal Brief did not contain the items required under 37 CFR 41.37(c).

Applicant respectfully appeals to the Board for review of the Examiner's final rejection.

(1) Real Party in Interest.

The real party in interest is the University of Iowa Research Foundation.

(2) Related Appeals and Interferences.

Application Serial No. 09/930,105, which claims priority to the instant application, is also currently being appealed to the Board for review of the Examiner's final rejection. There are no related interferences.

(3) Status of Claims.

Claims 2-5, 12-22, and 24 have been canceled. Claims 1, 6-11, 23, and 25 are pending and stand finally rejected. Applicant is appealing the final rejection of claims 1, 6-11, 23, and 25.

(4) Status of Amendments.

No Amendments have been have been filed subsequent to the Final Office Action.

(5) Summary of the Claimed Subject Matter.

The claimed subject matter relates to methods for normalizing the contractile response of vasculature in a patient in need of such normalization, wherein the vasculature has a vascular smooth muscle cell layer and a compromised endothelial cell layer, and wherein the methods include administering a pharmaceutically effective amount of a CLC3 blocker or a pharmaceutically acceptable salt thereof. The claimed subject matter also relates to methods for normalizing the contractile response of vasculature in response to norepinephrine in a patient in need of such normalization, wherein the vasculature has a vascular smooth muscle cell layer and a compromised endothelial cell layer, wherein the method includes administering a pharmaceutically effective amount of a CLC3 blocker or a pharmaceutically acceptable salt thereof. This subject matter is described throughout the specification, for example, at page 3, line 21 through page 12, line 16.

(6) Grounds of Rejection to be Reviewed on Appeal.

The issues being appealed are: (1) whether claims 1, 6-11 or 23 are unpatentable under 35 U.S.C. § 103(a) over U.S. Patent No. 6,197,789 (the Grainger *et al.* patent); and (2) whether claim 25 is unpatentable under 35 U.S.C. § 103(a) over the Grainger *et al.* patent in view of U.S. Patent No. 5,470,883 (the Stromberg patent).

(7) Argument.

A. Claims 1, 6-11 and 23 are patentable over the Grainger *et al.* patent

The Examiner alleges that claims 1, 6-11 and 23 are unpatentable over the Grainger *et al.* patent. The Examiner stated that the Grainger *et al.* patent teaches the use of tamoxifen to prevent or treat conditions characterized by inappropriate or pathological activity of endothelial cells. The Examiner also stated that the Grainger *et al.* patent teaches the use of tamoxifen to inhibit the activation of endothelial cells associated with vascular surgery, diabetes, hypertension, and coronary artery blockage. The Examiner further stated that the Grainger *et al.* patent teaches that procedural vascular traumas and pathologies such as atherosclerosis, myocardial infarction, and stroke can be

prevented by administration of tamoxifen. Thus, the Examiner concluded that it would have been obvious to one of ordinary skill in the art to modify the teaching of Grainger *et al.* and employ tamoxifen to normalize the contractile response of vasculature, since the teaching of "inhibiting contraction" encompasses "normalization," and since the effect of inhibition of contraction of vascular smooth muscle would "normalize" the contraction in patients as disclosed by Grainger *et al.*

(i) The Grainger *et al.* patent does not teach or suggest all elements of the claims.

The Grainger *et al.* patent fails to render claims 1, 6-11 and 23 obvious. First, the Grainger *et al.* patent does not teach or suggest all of the elements of the claims. Specifically, the Grainger *et al.* patent does not suggest using tamoxifen to normalize the contractile response of vasculature having a vascular smooth muscle cell (VSMC) layer and a compromised endothelial cell layer. The Examiner agreed with this assertion, as the Office Action of November 30, 2004 at page 3 states that "Grainger *et al.* do not expressly teach the normalization of contractile response set forth in claim 1."

The Grainger *et al.* patent discloses a therapeutic method for preventing or treating a cardiovascular or vascular indication characterized by a decreased lumen diameter, wherein a therapeutic agent that elevates the level of TGF-ß is employed (column 2, line 37 to column 3, line 2 and column 10, lines 44-46). The Grainger *et al.* patent also discloses that such an agent can inhibit the activity of a VSMC, such as proliferation, contraction, and migration (column 17, lines 41-48), as well as inhibit the "pathological" or "abnormal" activity of VSMC (column 3, lines 17-22 and column 6, lines 15-16), defined by Grainger *et al.* as "division, growth or migration of cells occurring more rapidly or to a significantly greater extent than typically occurs in a normally functioning cell of the same type, or in lesions not found in healthy tissues" (column 7, lines 61-65). The Grainger *et al.* patent does not, however, teach or suggest normalizing the contractile response of endothelially-compromised vascular smooth muscle.

Inhibition of VSMC contraction is not equivalent to normalization of smooth muscle cells. The Merriam-Webster online dictionary defines the term "inhibit" as "prohibit from doing something." In contrast, the Merriam-Webster online dictionary defines the term "normalize" as "reduce to a norm or standard." Figures 2 and 3 of Applicant's specification clearly depict the normalization response by showing that the contractile response of compromised VSM treated with tamoxifen was essentially the same as the contractile response of intact VSM, regardless of whether the intact VSM as treated with tamoxifen. Treatment of compromised VSM with tamoxifen did not

prohibit it from having a contractile response; the treatment instead reduced the contractile response to a normal level. Thus, even if the cited art suggested that tamoxifen could inhibit vascular smooth muscle cell contraction, there is nothing in the cited art to suggest that it can correct or normalize the contraction of endothelially-comprised VSMC. Therefore, the pending claims are not obvious in view of the Grainger *et al.* patent.

(ii) The Grainger *et al.* patent does not provide motivation to use tamoxifen to normalize contraction of compromised vasculature.

Further, the Grainger *et al.* patent does not provide motivation to use tamoxifen to normalize contraction of compromised vasculature. At no point does the Grainger *et al.* patent provide any evidence to indicate that tamoxifen has an effect on contractile VSMC (*i.e.*, mature, non-proliferative VSMC). A person of ordinary skill in the art would appreciate that contractile and proliferative VSMC serve different purposes, and thus have widely different properties (*see*, for example, Owens (1995) *Physiol. Rev.* 75:487-517). The Owens review teaches that mature VSMC proliferate at an extremely low rate and are almost completely geared for contraction, expressing a unique repertoire of contractile proteins, ion channels, and signaling molecules that clearly distinguish mature VSMC from any other cell type. In contrast, VSMC during vasculogenesis proliferate and produce matrix components of the blood vessel wall, and are not geared for contraction activities. Thus, the Owens review teaches that proliferative and contractile VSMC are functionally different. The Grainger *et al.* patent discloses only that tamoxifen has an effect on proliferative VSMC. Since the Grainger *et al.* patent fails to provide support for the notion that tamoxifen affects the contractile activity of mature VSMC, a person having ordinary skill in the art reading this reference would not have been motivated to use tamoxifen to normalize vasoconstriction of endothelially-compromised VMSC as recited in the present claims. As such, the Grainger *et al.* patent fails to render the presently claimed methods obvious.

(iii) The Grainger *et al.* patent does not provide a reasonable expectation of success for using a compound such as tamoxifen to normalize vasocontraction of compromised vasculature.

Moreover, the teachings of the Grainger *et al.* patent would not have provided a reasonable expectation of success for using a compound such as tamoxifen to normalize vasocontraction of compromised vasculature. The Grainger *et al.* patent fails to provide any evidence that tamoxifen can normalize vasoconstriction. The Grainger *et al.* patent discloses experimental data showing that

tamoxifen treatment of VSMC in culture can decrease cell proliferation and increase levels of TGF-beta, while tamoxifen treatment of mice on a high fat diet can reduce the formation of aortic lipid lesions. Thus, the Grainger *et al.* patent is focused on using compounds such as tamoxifen to inhibit proliferation of smooth muscle cells.

Further, Applicant's specification teaches that even after the Grainger *et al.* patent was filed, researchers did not know the effect of tamoxifen on VSM (*see*, for example, the sections of Applicant's specification at page 2, lines 12-15 and extending from page 26, line 21 to page 27, line 11). These sections disclose that at the time the inventor filed the present application, the inventor believed that tamoxifen treatment would not affect norepinephrine-induced contraction of normal vasculature (*i.e.*, vasculature having an intact endothelium). These sections further disclose that due to the lack of effect on normal VSM, the inventor did not previously examine the effect of tamoxifen on endothelially-compromised VSM. In addition, these sections of the specification disclose that Applicant's previous findings were published as Lamb and Barna (1998) *Am. J. Physiol.* 275:H151-H160, and Lamb and Barna (1998) *Am. J. Physiol.* 275:H161-H168. Thus, as of 1998, the effect of tamoxifen on normal VSM was uncertain. Due to this uncertainty, a person of ordinary skill reading the Grainger *et al.* patent would not have had a reasonable expectation that tamoxifen would normalize the contractile response of vasculature having a compromised endothelial layer.

In conclusion, since the Grainger *et al.* patent fails to teach or suggest all of the elements recited in the claims, and fails to provide either motivation or a reasonable expectation of success for using tamoxifen to normalize vasocontraction of compromised VSM, it does not render the present claims obvious. In light of the above, Applicant respectfully requests reversal of the Examiner's rejection of claims 1, 6-11, and 23 under 35 U.S.C. § 103(a).

B. Claim 25 is patentable over the Grainger *et al.* patent in view of the Stromberg patent

The Examiner alleges that claim 25 is unpatentable over the Grainger *et al.* patent as applied to claims 1, 6-11, and 23 above and further in view of the Stromberg patent. The Examiner stated that while the Grainger *et al.* patent does not teach that norepinephrine causes the contractile response of vasculature as set forth in claim 25, the Stromberg patent teaches a method of inhibiting or reversing the peripheral vasoconstrictive effect of norepinephrine by oral administration of tamoxifen citrate. Thus, the Examiner concluded that it would have been obvious to one of ordinary

skill in the art to employ tamoxifen to normalize the norepinephrine-induced contractile response of vasculature comprising a vascular smooth muscle cell layer and a compromised endothelial cell layer, because Stromberg teaches that tamoxifen is useful for reversing (normalizing) the vasoconstrictive effect of norepinephrine and because Grainger *et al.* teach that tamoxifen is useful for treating conditions characterized by inappropriate or pathological activity of vascular smooth muscle cells and endothelial cells.

Claim 25 recites a method to normalize the contractile response of vasculature in response to a vasoconstrictor agonist in a patient in need of such normalization. The cited patents fail to suggest such a method. As discussed above, "inhibiting" contraction is not the same as "normalization" of contraction. Neither the Grainger *et al.* patent nor the Stromberg patent suggests using tamoxifen to normalize the contractile response of vasculature in response to a vasoconstrictor agonist such as norepinephrine. In fact, the Office Action mailed on March 23, 2004, by which time both the Grainger *et al.* patent and the Stromberg patent were of record, included the following statement by the Examiner at pages 4 and 5:

> . . . there do not appear to be any examples or teachings in the prior art wherein a compound similar to the claimed compounds was administered to a subject to normalize the response to any vasoconstrictor (emphasis in original) . . . given the lack of . . . prior art regarding normalizing in response to a vasoconstrictor agonist (emphasis added) . . .

Given these statements, the Examiner appeared to believe, at least as of March 2004, that the prior art failed to teach or suggest using an agent such as tamoxifen to normalize contraction in response to a vasoconstrictor such as norepinephrine, as recited in claim 25. Since neither the Grainger *et al.* patent nor the Stromberg patent suggests using tamoxifen to normalize contraction, the combination of these references fails to render claim 25 obvious.

In light of the above, Applicant respectfully requests reversal of the Examiner's rejection of claim 25 under 35 U.S.C. § 103(a).

If necessary, please charge any additional fees or credit overpayment to Deposit Account 50-3503.

Respectfully submitted,

Fred S. Lamb

By his Representatives,

Viksnins Harris & Padys PLLP
PO Box 111098
St. Paul, MN 55111-1098
(952) 876-4091

Date: 6 October 2005

By: Ann S. Viksnins
Ann S. Viksnins
Reg. No. 37,748

"Express Mail" mailing label number: EV703160221US
Date of Deposit: October 6, 2005
It is certified that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicates above and is addressed to Mail Stop Appeal Brief - Patent, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

(8) Claims Appendix.

1. A method to normalize the contractile response of vasculature in a patient in need of such normalization, the vasculature comprising a vascular smooth muscle cell layer and a compromised endothelial cell layer, wherein the method comprises administering a pharmaceutically effective amount of a CLC3 blocker, or a pharmaceutically acceptable salt thereof.

6. A method of claim 23, wherein the compound administered is 1-p-∃-dimethylaminoethoxyphenyl-trans-1,2-diphenylbut-1-ene, or a pharmaceutically acceptable salt thereof.

7. A method of claim 23, wherein the patient has diabetes.

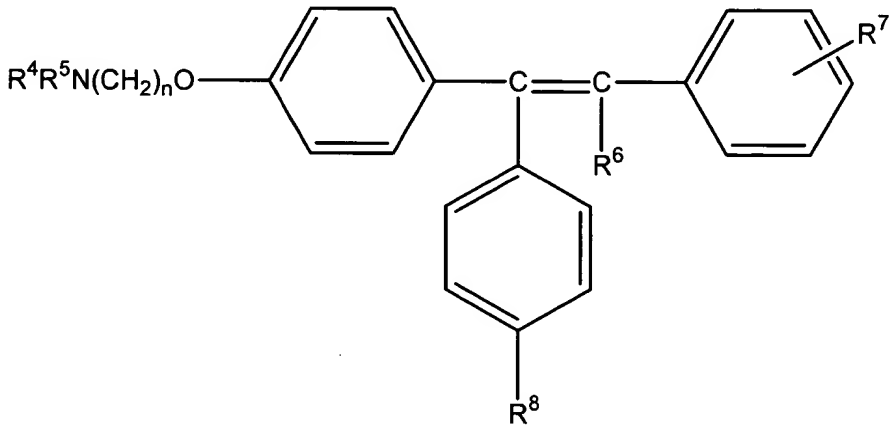
8. A method of claim 23, wherein the patient has had a surgical procedure.

9. A method of claim 23, wherein the patient has hypertension.

10. A method of claim 23, wherein the patient has coronary artery disease.

11. A method of claim 23, which further comprises administering a pharmaceutically-effective compound selected from the group consisting of: an anti-diabetes agent; an anti-hypertension agent; an anti-coronary artery disease agent; and an anti-restenosis agent.

23. A method of claim 1, wherein the CLC3 blocker is a compound of Formula I



wherein

either R^4 is H or a lower alkyl radical and R^5 is a lower alkyl radical, or R^4 and R^5 are joined together with the adjacent nitrogen atom to form a heterocyclic radical;

R^6 is H or a lower alkyl radical;

R^7 is H, halo, OH, a lower alkyl radical, or is a buta-1,3-dienyl radical which together with the adjacent benzene ring forms a naphthyl radical;

R^8 is H or OH; and

n is 2;

or a pharmaceutically acceptable salt thereof.

25. A method to normalize the contractile response of vasculature in response to a vasoconstrictor agonist in a patient in need of such normalization, the vasculature comprising a vascular smooth muscle cell layer and a compromised endothelial cell layer, wherein the method comprises administering a pharmaceutically effective amount of a CLC3 blocker, or a pharmaceutically acceptable salt thereof, and wherein the vasoconstrictor agonist is norepinephrine.

(9) Evidence Appendix.

A. U.S. Patent No. 5,470,883.

This document was entered by the Examiner in the Office Action mailed October 10, 2001.

B. U.S. Patent No. 6,197,789.

This document was entered by the Examiner in the Office Action mailed July 30, 2002.

C. Owens (1995) *Physiol. Rev.* 75:487-517.

This document was entered by the Examiner in the Office Action mailed November 30, 2004.

D. Definition of "inhibit", The Merriam-Webster Online Dictionary.

This document was submitted to the patent office with the Reply mailed February 28, 2005.

E. Definition of "normalize", The Merriam-Webster Online Dictionary.

This document was submitted to the patent office with the Reply mailed February 28, 2005.

F. Lamb and Barna (1998) *Am. J. Physiol.* 275:H151-H160.

This document was entered by the Examiner in the Office Action mailed October 10, 2001.

G. Lamb and Barna (1998) *Am. J. Physiol.* 275:H161-H168.

This document was entered by the Examiner in the Office Action mailed October 10, 2001.

PHYSIOLOGICAL REVIEWS
Vol. 75, No. 3, July 1995
Printed in U.S.A.

C

Regulation of Differentiation of Vascular Smooth Muscle Cells

GARY K. OWENS

Department of Molecular Physiology and Biological Physics, University of Virginia School of Medicine, Charlottesville, Virginia

Owens, Gary K. Regulation of Differentiation of Vascular Smooth Muscle Cells. *Physiol. Rev.* 75: 487–517, 1995.—The vascular smooth muscle cell (SMC) in mature animals is a highly specialized cell whose principal function is contraction. The fully differentiated or mature SMC proliferates at an extremely low rate and is a cell almost completely geared for contraction. It expresses a unique repertoire of contractile proteins, ion channels, and signaling molecules that are required for its contractile function and that when taken in aggregate clearly distinguish it from any other cell type. During vasculogenesis, however, the SMC's principal function is proliferation and production of matrix components of the blood vessel wall. Moreover, even in mature animals, the SMC retains remarkable plasticity, such that it can undergo relatively rapid and reversible changes in its phenotype in response to changes in local environmental cues normally required for maintenance of its differentiated state. A key to understanding SMC differentiation is to identify the key environmental signals and factors that induce or maintain the differentiated state of the SMC and to determine the molecular mechanisms that control the coordinate expression of genes encoding for proteins that are necessary for the contractile function of the SMC. The purpose of this review is to summarize our current knowledge of the regulation of SMC differentiation, with a particular emphasis on consideration of how this process is controlled during normal vascular development and how these control processes might be altered in vascular diseases such as atherosclerosis, which are characterized by marked alterations in the differentiated state of the SMC.

I. INTRODUCTION

Accelerated proliferation of smooth muscle cells (SMCs) is known to play an integral role in atherosclerotic lesion formation as well as postangioplasty restenosis and is a characteristic feature in arteries of hypertensive patients and animals (213, 234). There has thus been extensive interest in defining both positive and negative regulators of SMC growth, and many factors have been identified that may play a role in this process (213, 214, 234). In contrast, despite clear evidence that the differentiated state of the intimal SMC is altered within atherosclerotic lesions (89, 139, 141, 170), and that this is likely to play a key role in lesion development, relatively little is known regarding the mechanisms and factors that regulate changes in the differentiated state of the SMC in vascular disease. An understanding of the normal regulation of vascular SMC differentiation will also be critical to understanding congenital defects in vascular development, as well as vascular development in solid tumors, a key rate-limiting step in the pathobiology of most cancers (70). As such, there is considerable interest in understanding the cellular and molecular regulation of the differentiation of vascular smooth muscle cells.

The purpose of this review is to summarize our cur-

0031-9333/95 $3.00

rent knowledge of the mechanisms that regulate differentiation of vascular smooth muscle with an emphasis on consideration of developmental control events, as well as on alterations in differentiation control that occur in vascular disease. Because our understanding of the regulation of vascular SMC differentiation is in its infancy as compared with a number of other cell systems, I begin with a brief review of some of the general principles of differentiation control that have been established in studies of other cell systems that are likely to be applicable, at least in part, to differentiation control in smooth muscle.

II. MECHANISMS OF CONTROL OF CELLULAR DIFFERENTIATION: THE SKELETAL MUSCLE PARADIGM

Cellular differentiation is the process by which multipotential cells in the developing organism acquire those specific functions and properties that distinguish them from other cell types (see Ref. 55 for a review). This process has conventionally been subdivided into three stages: determination, differentiation, and maturation. Determination is the process by which multipotential cells in the developing embryo become committed to a particular cell lineage. Differentiation is the process by which cells that are committed to a particular cell lineage first manifest those cell-specific characteristics that distinguish that cell type. Maturation refers to the later stages of differentiation and is characterized by acquisition of further cell-specific properties ultimately resulting in the cellular phenotype characteristic of the mature organism. Although these are often considered to be distinct stages, they are really a continuum, with our ability to distinguish the stages being dependent on the extent of our knowledge of the cellular markers that characterize that stage and the molecular processes that control progression from one stage to another. Indeed, in most cases, the determination event can only be identified retrospectively from cell labeling studies, since by definition cells in this stage have not yet acquired cell-specific characteristics that allow them to be recognized. For purposes of simplicity in this review, we use the term *differentiation* to refer to the entire process by which committed but undifferentiated SMCs acquire their cell-specific phenotypes, recognizing that the immediate precursor to the differentiated SMC (i.e., a SMC myoblast) and the precise embryological origins of that cell have not, as yet, been clearly identified.

Despite the critical importance of considering differences between cell types, studies in other cells, principally skeletal muscle, have established a number of general principles of differentiation control that are likely to be applicable to differentiation control in multiple cell systems including vascular smooth muscle. Studies in skeletal muscle have demonstrated that differentiation involves continuous regulation rather than permanent activation or inactivation of genes, and several families of master regulatory genes that control skeletal muscle differentiation have been identified [see reviews by Olson (189), Weintraub et al. (285), and Blau and Baltimore (22)]. The first of these, MyoD, was isolated by subtractive hybridization approaches using an inducible differentiation system in which multipotential 10T1/2 mouse fibroblasts were converted to skeletal muscle through treatment with the DNA-hypomethylating agent 5-azacytidine (57, 267). MyoD and the related factors myogenin (67, 292), myf-5 (27), and MRF/herculin/myf-6 (208) encode transcriptional regulatory factors that are capable of converting a variety of cell lines, including smooth muscle (278), to skeletal myoblasts. The actions of these factors are mediated, at least in part, via the direct activation of a number of muscle-specific genes, including muscle creatine kinase, cardiac α-actin, myosin light chain, and troponin I [reviewed by Olson (189) and Weintraub et al. (285)]. They are part of a larger family of eukaryotic transcriptional regulators that contain a basic helix-loop-helix (HLH) motif that is involved in protein dimerization and DNA binding. Members of the MyoD family dimerize with ubiquitously expressed members of the HLH family such as E12, E47, and ITF and subsequently bind a consensus sequence (CANNTG), referred to as an E box, found in the promoters of many skeletal muscle genes and activate gene transcription. Results of transgenic and gene knockout experiments in mice have confirmed a vital role of the MyoD family members in control of skeletal muscle differentiation and have shown that their roles are somewhat redundant (reviewed in Refs. 107, 219, 284).

Another group of transcription factors referred to as the MEF2 proteins has also been shown to play a significant role in the myogenic cascade triggered by the MyoD family (53). Tontonoz et al. (272) have recently identified a novel HLH transcription factor, ADD1, that appears to play a role in the regulation of determination- and differentiation-specific gene expression in adipocytes. The preceding studies have thus established the presence of master regulatory genes that are involved in the continuous control of the differentiation program in skeletal muscle and adipocytes. Although it is likely that differentiation control genes analogous to MyoD are also expressed in smooth muscle, as yet no such factors have been identified.

III. CHARACTERISTICS OF DIFFERENTIATED SMOOTH MUSCLE CELLS

A. *Smooth Muscle Cells Are Multifunctional*

The principal function of the vascular SMC in mature animals is contraction, and the SMC has evolved a repertoire of appropriate contractile proteins, agonist receptors, ion channels, and signal-transducing molecules to carry out this specialized function. Indeed, the essence of understanding the control of differentiation of the SMC, as with any cell type, is to understand how the cell coordinately regulates the expression of those genes necessary for its specialized function. Although the SMC, like the majority of somatic cells in mammals, contains a complete set of genetic material, it expresses only a very small

number of the genes present. How is it determined which genes will be expressed, when, and at what levels?

Although the principal function of the mature SMC is contraction, this cell is also capable of a multitude of other functions that vary at different developmental stages, during vascular repair, and in vascular disease (234). For example, fully differentiated SMCs in mature blood vessels proliferate at extremely low rates and produce only small amounts of extracellular matrix proteins; these processes are greatly accelerated during development of the vascular system, during vessel remodeling, following vessel injury, and in atherogenesis. Indeed, the remarkable plasticity of the vascular SMC must be considered a necessary part of the SMC differentiation program that has evolved because it conferred a survival advantage to the organism.

The plasticity of the SMC differentiated state, however, has confounded efforts to understand the cellular and molecular mechanisms that control its differentiation; that is, a given SMC can acquire a broad spectrum of different phenotypes in response to different physiological or pathological stimuli. If an artery is injured, some SMC must be recruited to repair that injury, while at the same time the contractile function of the blood vessel must be maintained for normal cardiovascular homeostasis.

Each of the different phenotypic states of the SMC has somewhat different marker proteins that are characteristic of that state (considered in detail in sect. III*B*), and, as a consequence, presumably has differences in the mechanisms that regulate that particular differentiation program. This differs considerably from what occurs in cardiac and skeletal muscle that undergo terminal, and essentially irreversible, differentiation and that exhibit much more restricted cellular plasticity (23, 189). Control of differentiation in these cell types, while proving to be extremely complex, is nevertheless easier to study because of the stability associated with the terminally differentiated state. Thus key considerations in studying differentiation of SMC are *1*) to first establish which of the multiple biologically relevant SMC phenotypes is being studied, *2*) to identify a repertoire of marker proteins and cellular functions that characterize that phenotypic state and their temporal pattern of expression, and *3*) to develop appropriate experimental systems with which to determine the cellular and molecular mechanisms that control that process. It is critical to distinguish proteins that are characteristic of a given stage (or state) of SMC differentiation/maturation versus proteins that alone can serve as a definitive markers for identification of SMC lineages to the exclusion of all other cell types. Although it has been the goal of many investigators to identify the latter, at this time no marker strictly meets these criteria, with the possible exception of smooth muscle myosin heavy chain isoforms, SM-1 and SM-2 (1, 164, 216, 217). Indeed, given the multifunctionality of the SMC and the fact that it shares many of these functions with other cell types, such markers may not exist. At this time, clear identification of SMC and assessment of its state of differentiation must rely on more than one criteria, including expression of multiple SMC-selective proteins, the morphological and functional characteristics of the cell, and the cell's anatomic location.

This review focuses on consideration of the mechanisms that regulate the differentiated state of the fully contractile SMC, which is by far the most predominant phenotype found in the media of arteries of mature animals. Undoubtedly alternative phenotypes exist and play an important role in vascular pathologies [see sect. IX and a review by Schwartz et al. (234)]. However, an understanding of the abnormal control of differentiation in disease states will only be possible once we understand how differentiation is normally regulated in mature fully contractile vascular SMCs.

B. Markers of Differentiated Smooth Muscle Cells

1. Smooth muscle α-actin

The contractile proteins represent logical candidates for use in studying differentiation of contractile SMCs. Indeed, mature vascular smooth muscle has been shown to express unique isoforms of a variety of contractile proteins that are important for their differentiated function. The first of these identified was smooth muscle α-actin, which is one of six isoactins expressed in mammalian cells (276). All are products of separate genes, although they share an extremely high degree of homology in the protein-coding regions (276). Mature fully differentiated SMCs express four actin isoforms including smooth muscle α-actin, nonmuscle β-actin, nonmuscle γ-actin, and smooth muscle γ-actin (77, 197). The most abundant of the actin isoforms in mature fully differentiated vascular smooth muscle is smooth muscle α-actin, which is also the single most abundant protein in SMCs making up 40% of total cell protein and over 70% of the total actin (69). There is some indirect evidence that actin isoform diversity may be of functional significance. For example, several workers have reported selective localization of isoactin proteins as well as mRNAs within the cell (231, 261), and there is evidence for differential affinity of actin isoforms for actin-binding proteins (126). However, there is little direct evidence that there are major functional differences between the different actin isoforms (62, 204). Nevertheless, the high smooth muscle α-actin content of vascular SMCs is required for their high force-generating capability (174, 197). There is also clear evidence that expression of the different actin genes is differentially regulated at the transcriptional level (227). As such, it is reasonable to suggest that quantitative differences in the level of smooth muscle α-actin expression may be of equal or greater importance for differentiation/maturation of SMC than qualitative shifts in which actin isoforms are expressed.

Results of early studies indicated that smooth muscle α-actin was expressed exclusively by SMCs and SMC-related cells such as pericytes (182) and juxtaglomerular

cells (90). However, it is now known that it is transiently expressed by a variety of mesodermally derived cells during development, tissue repair, and neoplastic growth (41, 54, 225). For example, smooth muscle α-actin is transiently expressed in the early stages of differentiation of both cardiac and skeletal myocytes (221, 291), as well as in myofibroblasts in healing wounds (54) and tumors (41). Its expression can also be induced in a number of non-SMCs in culture, including microvascular endothelial cells and myofibroblasts by treatment with transforming growth factor-β (TGF-β) (3, 160). Thus smooth muscle α-actin expression alone does not provide definitive evidence for SMC lineage. However, its expression in adult animals is highly tissue specific under normal circumstances (77, 291). Moreover, as discussed in detail in section VIII, there is clear evidence for cell type-specific regulation of transcription of this gene, i.e., although a number of different cell types can express the gene under certain circumstances, regulation of its expression is quite different between different cell types, thus making it a very useful gene with which to study the molecular regulation of the differentiation program in vascular smooth muscle (18, 35, 71).

Smooth muscle α-actin is also the first known marker of differentiated SMCs that is expressed during vasculogenesis (64, 122, 166). It is first detected in the presumptive SMCs that first envelope the dorsal aortas at stage 12 (*day 2* of development) in chicken (64) and quail embryos (122) (see also sect. IV). Significantly, at this stage, smooth muscle α-actin expression was limited to those presumptive SMCs that were in direct contact with the dorsal aortic endothelial cells and was not observed in surrounding mesodermal cells. Furthermore, although smooth muscle α-actin is detectable very early in the developing vasculature, major increases in its level of expression occur during development (9, 140, 197). Nonmuscle β-actin is the major actin expressed in aortic SMCs of 3-day-old rats, and <60% of the medial SMCs at this age show detectable smooth muscle α-actin expression as determined by immunocytochemical labeling using a high-affinity smooth muscle α-actin monoclonal antibody (9, 197). This indicates that differentiation/maturation of SMC within a given blood vessel is not synchronous during development. Smooth muscle α-actin does not become the predominant actin isoform in rat aortic SMCs until nearly 30 days of age. Similar postnatal increases in smooth muscle α-actin expression have also been observed in a number of other species including pigs (69) and humans (184, 218). Taken together, the preceding results indicate that smooth muscle α-actin is not only the earliest known marker of differentiated SMCs expressed during development of the vasculature but also undergoes major increases in its level of expression late in vascular development.

2. *Smooth muscle myosin heavy chains*

Mature vascular SMCs also express a number of cell-specific/selective isoforms of myosin (1, 73, 180, 216, 217, 286). Myosin is an essential component of the contractile system that is present in all muscle and nonmuscle cells. It is a hexamer consisting of two myosin heavy chains (MHC), a pair of 17-kDa nonphosphorylatable alkali light chains (also designated MLC-1 and MLC-3), and a pair of regulatory (phosphorylatable) 20-kDa light chains (MLC-2). Myosin regulatory light chain from vertebrate smooth muscle as well as nonmuscle cells plays a key role in the regulation of smooth muscle contraction and nonmuscle cell motility via Ca^{2+}/calmodulin-dependent phosphorylation catalyzed by myosin light-chain kinase (see Refs. 175, 253 for a review). Multiple isoforms of all of these subunits of myosin have been found, and the expression of the isoforms is differentially regulated in a tissue-specific and developmental stage-related manner (73, 253).

Vascular SMCs express at least three smooth muscle variants as well as two nonmuscle variants of the heavy chain (1, 180, 216, 286). Kawamoto and Adelstein (133) have classified the 196-kDa nonmuscle-type MHC as NMHC-A and the 198-kDa nonmuscle MHC as NMHC-B. The NMHC-B appears to be identical to the 198-kDa MHC designated SMemb that is expressed in developing rabbit embryonic aortas, intimal SMCs of animals with experimental atherogenesis, human atherosclerotic lesions, and cultured SMCs (6, 73, 143, 144). The SMC variants were originally identified on the basis of their differential migration on porous sodium dodecyl sulfate (SDS) polyacrylamide gels as well as their reactivity with myosin antibodies on Western blots (6, 216, 217). Results of these studies demonstrated the presence of 204- and 200-kDa MHC proteins that have been identified as SM-1 and SM-2, respectively. Subsequent studies by Periasamy and co-workers (180) demonstrated that the smooth muscle variants SM-1 and SM-2 are produced by alternative splicing of a gene that is unique from the genes encoding nonmuscle, skeletal, or cardiac MHC isoforms. The SM-2 isoform contains 9 amino acids not contained in SM-1 that are encoded by a unique 39-nucleotide exon at the COOH-terminal, whereas the SM-1 isoform contains a longer COOH-end containing 43 amino acids. The functional significance of these differences in the MHC tail is not known.

More recent studies have identified additional isoform diversity of SM-1 in the S1 head region (5, 135, 286). These isoforms have been designated SM-1A and SM-1B. Their mRNAs are completely identical in their coding regions except that the SM-1B isoform contains an insert of 21 nucleotides, encoding 7 amino acids in a region near the ATP binding site in the myosin head. S1 nuclease protection assays demonstrated that SM-1A and SM-1B mRNAs are coexpressed in all smooth muscle tissues, although the proportion of the two mRNA differs markedly between tissues. The SM-1A form predominates in most smooth muscle tissues including vascular SMCs, whereas SM-1B predominates in intestinal and urinary bladder SMCs. Kelley et al. (135) found that the presence of the seven-amino acid insert in SM-1B correlated with a higher velocity of movement of actin filaments in vitro and a higher actin-activated Mg^{2+}-adenosinetriphosphatase (ATPase) activity compared with SM-1A myosin, sug-

gesting that the presence of the insert in SM-1B may be of functional importance and may contribute to differences in contractile properties between different SMC tissues [for a comprehensive review of this area, see the recent review by Somlyo (253)].

Expression of smooth muscle MHC isoforms has been extensively scrutinized and shows a high degree of SMC specificity in both mature and developing organisms (1, 73, 74, 87, 144, 164, 216). Indeed, SM-1 and SM-2 may be the most rigorous markers for identification of differentiated SMC because, thus far, they show the highest degree of cell specificity of any of the known markers of differentiated SMC. There is evidence suggesting that smooth muscle MHCs can be expressed in subconfluent bovine aortic endothelial cells in culture (but not confluent cells or endothelial cells in vivo) (25), as well as in myofibroblasts and myoepithelial cells in normal and malignant human breast tissue (148). However, the results of these studies must be viewed with some skepticism, since no Western blot analyses were provided to document the specificity of the smooth muscle MHC antibody employed, and we have found that the principal antibody employed in these studies (which is now commercially available from Sigma; hSM-V, catalog no. M7754) shows cross-reactivity with a 198- to 200-kDa nonmuscle MHC (presumably NMHC-B or SMemb, see Refs. 133, 143) that is present in endothelial cells and migrates very closely with SM-2, but is not recognized by our smooth muscle MHC-specific monoclonal antibodies (206; M. M. Thompson and G. K. Owens, unpublished data). This cross-reactivity must also be taken into account in interpreting results of other immunolabeling studies done using this antibody (e.g., Refs. 25, 73, 74, 87), since this likely resulted in some erroneous conclusions regarding the cellular distribution and the temporal sequence of expression of smooth muscle MHC isoforms during SMC differentiation/maturation.

Expression of the various MHC isoforms in SMC shows extensive developmental regulation (1, 144). Based on immunocytochemical studies and Western blot analysis with SM-1 and SM-2 specific antibodies as well as S1 nuclease protection assays to distinguish SM-1 versus SM-2 mRNAs, Kuro-o et al. (144) demonstrated that vascular SMCs from adult rabbits expressed both SM-1 and SM-2 MHCs. In contrast, fetal SMCs expressed the 200-kDa nonmuscle and SM-1 MHC isoforms but not SM-2. They did express a MHC isoform that comigrated with SM-2 on polyacrylamide gels but that did not react with the SM-2 specific antibody. This MHC isoform was subsequently shown by this same group to be a nonmuscle MHC isoform expressed in a variety of non-SMC tissues and was designated SMemb by Kuro-o et al. (143) and nonmuscle MHC-B by Kawamoto and Adelstein (133). Of considerable interest, SM-2 expression was first detectable in vascular SMCs 10 days postnatally and gradually increased to adult levels, indicating that it is a marker of a later stage of differentiation/maturation than SM-1 MHC, smooth muscle α-actin, h-caldesmon, calponin, and many of the other markers of differentiated SMCs (144). Aikawa et al. (1) found that similar developmental changes in MHC expression occur in humans. They reported that SM-1 as well as nonmuscle MHCs were expressed in fetal arteries of the early gestational stage, whereas SM-2 was upregulated during late fetal and postnatal development.

In recent studies, Miano et al. (164) utilized in situ hybridization and ribonuclease protection assays to assess the temporal and spatial pattern of SMC differentiation during mouse development. Although their assays did not distinguish between SM-1 and SM-2, their results demonstrated that smooth muscle MHC expression was completely restricted to smooth muscle tissues and was first evident in the early developing aorta at 10.5 days postcoitum. No expression was demonstrated beyond the aorta and its arches until 12.5–13.5 days postcoitum when smooth muscle MHC mRNA appeared in smooth muscle cells of the developing gut as well as in peripheral blood vessels. No smooth muscle MHC transcripts were ever detected in developing brain, heart, or skeletal muscle, except within blood vessels within these tissues. Taken together, the preceding results establish that smooth muscle MHC is a highly specific marker of the SMC lineage. However, further scrutiny of smooth muscle MHC expression may identify exceptions. This being the case, clear identification of a cell as a SMC should rely on analysis of multiple SMC differentiation markers, including in the case of in vivo studies, the anatomic location of the cell in question.

3. *Myosin light chains*

Two smooth muscle isoforms of the alkali 17-kDa light chain have been identified in vascular smooth muscle that are electrophoretically distinguishable (68, 106, 112, 129). The more acidic of these has been designated LC_{17a}, whereas the more basic form is designated LC_{17b} (112, 129, 157). Both the LC_{17a} and LC_{17b} isoforms are expressed in vascular SMCs, whereas SMCs of the gastrointestinal tract as well as fibroblasts express almost exclusively the LC_{17a} form (112). Both isoforms, however, are expressed to some extent in both smooth muscle and nonmuscle cells. Both LC_{17a} and LC_{17b} are the products of alternative gene splicing that leads to alterations in five of nine COOH-terminal amino acids (106, 152, 178). A 23-kDa light chain has also been detected in embryonic chicken gizzard that is downregulated after hatching (131, 265). This embryonic myosin light chain (MLC) isoform is also transiently expressed in embryonic sarcomeric tissues (134). At this time there are no published reports of this isoform being expressed in vascular SMCs, and because it has not been cloned, it is unknown what degree of homology it shares with other MLCs.

The 17-kDa light chains are believed to be necessary for modulating the affinity of myosin for F-actin as well as actin-activated ATPase activity (reviewed in Ref. 129). There is also some correlative evidence that different ratios of the two 17-kDa variants affect the enzymatic and functional properties of the actomyosin complex in that

smooth muscle tissues enriched in the LC_{17b} isoform show a lower actomyosin ATPase activity (112) and diminished maximal shortening velocity (157) compared with those containing more LC_{17a}.

Multiple isoforms of the phosphorylatable 20-kDa regulatory MLC have been described in arterial smooth muscle (reviewed in Ref. 253). This includes a "smooth muscle" regulatory light chain (also designated $L_{20\text{-}A}$) and a "nonmuscle" isoform (also designated $L_{20\text{-}B}$). Despite their names that imply cell-specific expression, both are expressed in multiple smooth muscle as well as nonmuscle cells and tissues (253, 268). For example, platelets contain approximately equal amounts of the smooth muscle and nonmuscle 20-kDa MLC isoforms. There are, as yet, no reports of there being isoformic variants that affect function.

4. *Calponin and SM-22α*

Calponin is a 28- to 34-kDa protein that interacts with F-actin and tropomyosin in a Ca^{2+}-independent manner and with calmodulin in a Ca^{2+}-dependent manner and has been found to inhibit actin-activated Mg^{2+}-ATPase activity of myosin in vitro (reviewed in Ref. 289). On the basis of these properties, it has been postulated to function as a regulator of SMC contraction. However, the mechanism of actin-linked regulation via calponin is by no means clear, either with regard to the possible role of phosphorylation or with respect to the reported additional involvement of caldesmon (252, 290). Multiple isoforms of calponin have been identified by molecular cloning (185, 264). Two of the encoded isoforms that were originally identified in avian species are denoted α- and β-calponin. They have 292 and 252 amino acids and M_r values of 32,333 and 28,127, respectively, and appear to be derived by alternative splicing in that the nucleotide sequences are identical except for a 120-bp insert in α-calponin that encodes a 40-amino acid segment corresponding to residues 217–256. Mammalian homologues of α- and β-calponin have also been identified that have been designated h_1-calponin (for high molecular weight) and l-calponin (for low molecular weight), respectively (2, 258). A novel calponin variant, designated h_2, has been recently identified which shares a high degree of homology with α- and β-calponin (or h_2- and l-calponin) except for a short highly acidic domain in the COOH-terminal (2, 258). h_2-Calponin is expressed by both SMCs as well as non-SMCs, and on the basis of sequence analysis appears to be the product of a separate gene from that encoding h_1- or l-calponin (258). The functional significance of calponin isoform diversity has yet to be elucidated. A 22-kDa protein, designated SM-22α, has also been identified in SMCs which contains sequence motifs that are homologous to calponin (185). However, unlike calponin, there is no evidence that it binds to any contractile protein, and at present, its function is unknown.

In adult organisms, the expression of the α- and β-calponin (h_2- and l-) isoforms and SM-22α appears to be restricted almost exclusively to smooth muscle. Shanahan et al. (241) found high levels of calponin and SM-22α mRNA in aorta, bladder, vas deferens, and uterus but not in kidney or brain in adult rats. Gimona et al. (82) observed no calponin immunoreactivity in extracts of chicken skeletal muscle, kidney, liver, and spleen. Takahashi et al. (263) found no evidence for expression of calponin in bovine atria, ventricles, or brain cortex, but did observe a 36-kDa immunoreactive protein in bovine adrenal medulla and cortex. Calponin-immunoreactive proteins have also been detected in bovine platelets, human umbilical vein endothelial cells, and fibroblasts (17, 266), although it remains to be established unequivocally that these are indeed calponins. One possibility is that these "calponin"-immunoreactive proteins correspond to h_2-calponin, which is known to be expressed in a variety of non-SMC tissues (2, 258).

Immunolabeling studies have shown that calponin and SM-22α expression is developmentally regulated in SMCs, although in most cases it is not clear which specific calponin isoform is being expressed. Calponin and SM-22α expression is first detectable in the dorsal aortas of the chick on *days 4–6* of embryonic development (64), thus making it one of the earliest markers of differentiated SMC (see Fig. 1). Likewise, Frid et al. (74) found evidence for developmental regulation of calponin in vascular SMCs in humans. They (74) observed that major increases in calponin expression occurred relatively late in development (i.e., >22–24 wk of gestation). However, consistent with observations of Duband et al. (64), low levels of calponin were also detectable in the 8- to 10-wk-old fetus, the earliest developmental time point studied.

5. *Caldesmon*

Caldesmon is a major calmodulin and actin-binding protein that is found in smooth muscle and nonmuscle cells (252). Caldesmon inhibits superprecipitation and actomyosin Mg^{2+}-ATPase activity in vitro (179). It also inhibits movement of actin filaments by myosin in in vitro motility assays (251), and exogenously added caldesmon relaxes permeabilized smooth muscle fiber preparations (179, 252). Whereas the preceding observations suggest that caldesmon plays a role in regulation of contraction, direct experimental evidence for this in intact smooth muscle tissues is currently lacking.

Two isoforms of caldesmon can be discerned by their mobility on SDS polyacrylamide gels: h-caldesmon (M_r of 120,000–150,000) and l-caldesmon (M_r of 70,000–80,000) (252), although their predicted molecular weight based on sequence is somewhat less (ca. 88,743 and 58,844, respectively) (109). On the basis of both Northern and Southern analyses and sequence comparisons, h- and l-caldesmon appear to be generated from a single gene via alternative splicing (121, 252). The two isoforms show markedly different cell and tissue distributions. The h-caldesmon isoform is abundantly expressed in differentiated smooth muscle, whereas l-caldesmon is found in non-

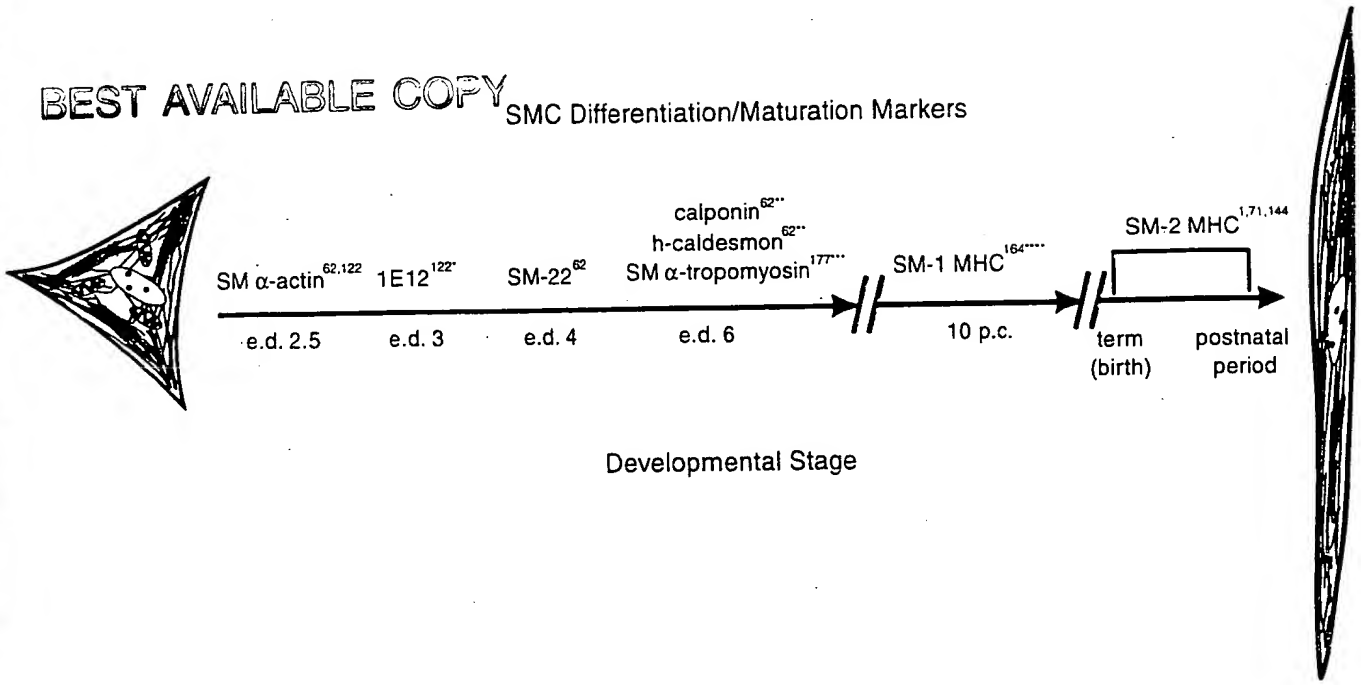


FIG. 1. Ontongeny of expression of smooth muscle cell (SMC) differentiation markers in developing vascular SMCs. Results shown illustrate earliest developmental stage that marker has been detected. To avoid confusion, I have not cited published reports that did not adequately distinguish non-smooth muscle (SM) versus SM isoforms of proteins that serve as SMC differentiation markers (e.g., SM α-tropomyosin versus skeletal or nonmuscle α-tropomyosin, h- vs. l-caldesmon, γ-vinculin vs. α-, α'-, and β-vinculin) or did not comprehensively examine when in development the marker is first expressed. * 1E12 is a monoclonal antibody recently developed against 10-day-old embryonic quail dorsal aorta by Hungerford and Little. (122). 1E12 recognizes an as yet unidentified antigen that appears to be specific for SM and is first detectable in presumptive SMCs that surround dorsal aortic endothelium at embryonic *day 3*. ** Frid et al. (74) reported that major increases in expression of calponin and h-caldesmon occurred very late in development of humans (>22–24 wk of gestation). However, both calponin and h-caldesmon were detectable in the 9- to 10-wk-old human fetus, the earliest developmental stage examined. Glukhova and co-workers (87, 88) also reported late expression of h-caldesmon and metavinculin in humans with levels detectable but low in the 10-wk-old fetus. To my knowledge, developmental changes in expression of metavinculin and γ-vinculin (a SM-selective isoform) have not been systematically characterized in other species. *** Study by Muthuchamy et al. (177) was based on analysis in whole mouse embryos and did not resolve SM α-tropomyosin in specific tissues. For purposes of this figure, I have assumed (unproven) that expression occurred in vascular SMCs. **** Studies of Miano et al. (164) relied on in situ hybridization with a probe that did not distinguish SM-1 and SM-2 myosin heavy chain (MHC) isoforms. However, on the basis of immunolabeling studies in rabbits (139) and humans (1) showing that SM-1 expression occurs much earlier that SM-2, it is likely that earliest SM MHC isoform detected by Miano et al. (164) was SM-1. e.d., Embryonic day (chicken or quail); p.c., days postcoitus (mouse).

muscle tissue and cells as well as immature SMCs (252, 275). Neither caldesmon isoform is detectable in adult skeletal and cardiac muscle. However, h-caldesmon is expressed in BC_3H1 skeletal myoblasts following serum depletion and density-dependent differentiation (275). BC_3H1 cells, which were originally derived from a mouse brain tumor, were at one time thought to be of a smooth muscle lineage based on observations that they were contractile, expressed smooth muscle α-actin, and were mononucleate. However, there is now compelling evidence that this is not the case, in that the cell expresses a multitude of skeletal muscle-specific proteins and most likely represents a fusion defective skeletal myoblast cell line (269). Whereas further studies are needed to determine whether h-caldesmon is also transiently expressed by skeletal myoblasts in vivo, it is clear that h-caldesmon expression, like smooth muscle α-actin, smooth muscle MLCs, and calponin is not strictly limited to SMCs. As such, it cannot be used as an unambiguous marker for identification of SMC lineages to the exclusion of all other cell types.

Expression of caldesmon is developmentally regulated in vascular as well as nonvascular smooth muscle. Duband et al. (64) first detected h-caldesmon in the dorsal aorta on embryonic *day 6* in the chick. In developing gizzards, l-caldesmon was detected in 10-day-old chick embryos (the earliest time point examined), whereas h-caldesmon was first detectable between 10 and 13 days of development (275). Expression of h-caldesmon increased progressively from *day 13* in parallel with increases in desmin, myosin, and β-tropomyosin. In contrast, expression of l-caldesmon decreased progressively to undetect-

able levels by 3 days posthatching. Koteliansky and coworkers (74, 87) examined changes in l- and h-caldesmon in human aortic SMCs during development based on immunohistochemical staining with monoclonal antibodies for each of these proteins, as well as by gel electrophoretic analyses. Results demonstrated that aortic SMCs from 8- to 10-wk-old fetuses expressed very low levels of h-caldesmon relative to l-caldesmon. Major increases in h-caldesmon expression in aortic SMCs occurred relatively late in development, including a nearly sixfold increase in the ratio of h- to l-caldesmon between 10- and 24-wk-old fetuses. Taken together, these results indicate that h-caldesmon may be a marker of a later SMC differentiation/maturation stage than smooth muscle α-actin but an earlier marker than SM-2 MHC (see Fig. 1).

6. *Vinculin and metavinculin*

Vinculin is a 117-kDa cytoskeletal protein associated with membrane actin filament attachment sites of cell-cell and cell-matrix adherens-type junctions (79, 200). There is remarkable heterogeneity of vinculin in the form of antigenically indistinguishable isoelectrophoretic variants, some of which show tissue selective expression (12, 142). These include three isoforms designated α, α', and β that are found in all cell types and a γ-form that shows selective expression in cardiac and smooth muscle (12). In addition to these isovinculins, smooth, cardiac, and skeletal muscle tissues were found to express a protein of 150 kDa that was antigenically related to vinculin, denoted metavinculin (88, 245). Two isoforms of metavinculin have been described denoted α- and β-metavinculin. In smooth muscle, both vinculin and metavinculin are located in F-actin membrane attachment sites of dense plaques (79, 200). Both α- and β-metavinculin show tissue-selective expression, with reports thus far showing expression only in smooth and cardiac muscle (12). No information is available as to when γ-vinculin and α- and β-metavinculin are first expressed during differentiation of vascular SMCs. However, developmental increases in expression of vinculin have been shown to occur postnatally in the human aorta (87). As such, the α- and β-metavinculins and γ-vinculin may be useful markers with which to study differentiation/maturation, although additional studies are needed in this area. It should be noted, however, that the SMC forms of vinculin and metavinculin are produced by alternative splicing from a single gene, thus making this gene family useful for studying posttranscriptional rather than transcriptional control of SMC differentiation/maturation (29, 167).

7. *Tropomyosin*

Tropomyosins are rodlike proteins that are usually found in tight association with actin filaments in muscle and nonmuscle cells (28, 149, 161). In skeletal and cardiac muscle, tropomyosins play a central role in regulation of contraction through mediation of the calcium response of the troponin complex to actin filaments (66). In contrast, the physiological role of tropomyosins in smooth muscle and nonmuscle cells is poorly understood, due in part to the fact that these cells lack troponin (28, 161). It has been suggested based on biochemical observations showing association of tropomyosin with actin that it may be involved in stabilization of actin filaments, although direct evidence for this is lacking.

Multiple isoforms of tropomyosin have been detected in muscle and nonmuscle cells at both the protein and mRNA levels (116, 119, 287). These are the products of an extremely complex (and often confusing) regulatory system involving at least four different genes, each encoding multiple muscle and nonmuscle isoforms through alternative splicing (see Ref. 100 for a review). Vascular SMCs express several nonmuscle as well as smooth muscle tropomyosin isoforms (69, 116, 220, 287). Smooth muscle α-tropomyosin is a product of differential mRNA splicing from a single α-tropomyosin gene and is unique from striated and nonmuscle α-tropomyosin isoforms by virtue of expression of exon 2 that encodes for amino acids 39-80 of the protein (220, 287). Expression of the smooth muscle α-tropomyosin isoform appears to be limited to SMCs, at least in adult organisms (287). However, the tissue distribution of smooth muscle α-tropomyosin has not been extensively scrutinized due in part to the lack of isoform-specific antibodies. Muthuchamy et al. (177) demonstrated that smooth muscle α-tropomyosin transcripts were first detectable as early as 4.5–6 days postcoitum in extracts of whole mouse embryos then increased with developmental age, with large increases in expression occurring between 8.5 and 10 days postcoitum. However, no data were presented regarding what specific cell types in the embryo were expressing these transcripts. These workers did observe expression of smooth muscle α-tropomyosin transcripts in undifferentiated embryonic stem cells and at all stages of embryoid body development in vitro. As such, smooth muscle α-tropomyosin is probably not useful as a SMC lineage marker for developmental studies. However, the fact that its expression in adult animals is restricted to SMCs and is developmentally regulated indicates that it is useful for assessing the relative state of differentiation/maturation of vascular SMCs.

8. *Intermediate filament proteins*

The principal intermediate filament (i.e., 7- to 11-nm filaments) proteins expressed by vascular smooth muscle are desmin and vimentin (77, 166, 249). Cytokeratin has also been detected in immature SMCs during development as well as in intimal atherosclerotic lesions (7). The proportion of desmin versus vimentin varies between different smooth muscle tissues and between different blood vessels within the vasculature. There also appear to be some species differences in the relative proportions and/or distribution of these two proteins. Vascular smooth muscle in the aorta and other large conduit vessels con-

tains predominantly vimentin rather than desmin (77), whereas the intermediate filaments in SMCs of smaller arteries and arterioles as well as gastrointestinal and uterine SMCs contain predominantly desmin (77, 166, 181). Gabbiani and co-workers (77, 218) have also presented evidence for heterogeneity in expression of desmin and vimentin within individual SMCs within the normal aortic media, with some cells expressing exclusively vimentin and others expressing both of these intermediate filament proteins.

Vimentin is expressed in a wide variety of muscle and nonmuscle cell types and is expressed very early in the developing embryo before the formation of the vasculature (14, 230). As such, it is of limited usefulness as either a marker of SMC lineage or for assessing the differentiation/maturation state of the SMC. Desmin expression is largely, but not exclusively, expressed in muscle cells, including cardiac and skeletal muscle, as well as smooth muscle (14, 230). Desmin expression is developmentally regulated in skeletal and cardiac muscle showing marked increases in expression during myogenesis (154). Similarly, desmin expression is developmentally regulated in the SMCs of the gut and urogenital tracts of the chicken (14) and mammals (72). However, surprisingly, there is a relative paucity of information regarding whether desmin expression is developmentally regulated in vascular smooth muscle, although it does not appear to be expressed early in development of the great vessels in the mouse (230). However, it should be noted that in rodents, desmin is also expressed at low levels in these vessels in mature animals (77). Taken together, studies indicate that desmin is clearly not a specific marker of the SMC lineage but may be useful in assessing the relative state of differentiation/maturation of vascular SMCs within certain, but probably not all, blood vessels.

9. *Integrins*

Establishment of appropriate cell-cell and cell-matrix contacts is critical for transduction of mechanical forces in smooth muscle tissues and, as such, is of key importance in development of the vasculature. Integrins are a group of nonconvalently associated heterodimers of α- and β-subunits that function in cell-to-cell and cell-to-matrix adhesions (for a review, see Ref. 123). Integrins have been classified according to their β-subunits, each which may associate with different α-subunits. The extracellular domains of the integrins mediate cell adhesion to extracellular matrix and basement membrane components, whereas the cytoplasmic domains interact with actin filament-associated proteins. Multiple α- and β-subunits have been identified that are widely distributed on most cell types. In general, integrins are not cell type specific. However, their expression is highly developmentally regulated in a cell type- and function-dependent manner, and they are known to play an important role in the differentiation of many cell types (123).

Relatively few studies have examined developmental changes in integrin expression in vascular SMCs. Glukhova et al. (86) studied the distribution of laminin variant chains, a major component of the extracellular matrix in blood vessels, and laminin-binding β_1-associated α-integrin subunits in human arterial SMCs during development using immunofluorescence methods. They found that during maturation of vascular SMCs in humans, there was a switch in expression of both laminin as well as integrin subunits. In aortic media of 10-wk-old fetuses, B1-, B2-, and A-laminin chains were found. At 27 wk, the S chain appeared and eventually replaced the B1-chain in the major part of the adult aortic media. Of the four β_1-associated α-integrin subunits that have been shown to recognize laminin (α_1, α_2, α_3, and α_6), only α_1 was seen in vascular SMCs in fetal arteries. The α_3-subunit appeared in aortic media only in the late fetal period. In adult arterial media, α_1 and α_3 were the major potential laminin-binding integrins. The spatial-temporal distribution of laminin and integrin variants in developing versus adult SMC suggests that they may play an important role in regulation of the differentiation of vascular SMCs. Duband et al. (63) found that α_1-integrin exhibited a restricted pattern of expression in adult avian tissues, being detected only in smooth muscle and endothelial cells, although it was detected in both the central and peripheral nervous systems and in striated muscles during development. Taken together, the preceding results suggest that studies of integrin expression may provide a useful index with which to assess the state of differentiation/maturation of the vascular SMCs. However, further studies are needed to define the specific integrin subunits involved, their temporal patterns of expression, and their function in developing vascular smooth muscle.

10. *Ion channels and receptors*

Vascular SMCs express a large repertoire of ion channels and membrane receptors that are critical in influencing the cells' contractile behavior through effects on its electrical activities and sensitivity to stimulation by hormones, neurotransmitters, and contractile agonists (see Refs. 125 and 250 for a review). The specific repertoire of ion channels and receptors that are expressed varies widely between different vascular beds and is a key determinant of the contractile responsiveness of the SMC. Most of these receptors and ion channels are not expressed in multipotential cells that give rise to SMCs, but rather appear during differentiation/maturation of the SMCs (20, 65). As such, they are logical candidates for use as SMC differentiation markers. With just a few possible exceptions, the ion channels and receptors expressed by vascular smooth muscle are also expressed by many other cell types (11, 125, 250). Moreover, there is little information available as to when they are first expressed during vasculogenesis, although there is evidence for developmental regulation of contractile agonist receptor levels in vascular SMCs.

Expression of angiotensin II receptor subtypes has

been shown to be developmentally regulated in vascular smooth muscle (96, 282). Whereas aortic SMCs in adult rats express almost exclusively the AT_1-type angiotensin II receptor, a major portion of the angiotensin II receptors in the fetal rat aorta (embryonic *day 18*) is of the AT_2 type (282). Similar changes have been reported to occur during late fetal development in human renal arteries by Grone et al. (96). There is also evidence for postnatal developmental regulation of a number of other contractile agonist receptors in vascular SMCs including muscarinic M_1, M_2, and M_3 receptors (37); vasopressin V_1 and V_2 receptors (190); α_1-adrenergic receptors (242); and β-adrenergic receptors (232). No studies, however, have been reported indicating when these contractile agonist receptors are first expressed during embryonic development of vascular SMCs.

Hart et al. (105) have presented evidence suggesting that colonic SMCs express a unique K^+ channel, designated CSMK1, that is expressed in colonic SMCs and brain but not in the portal vein, renal artery, uterus, or heart. To my knowledge, no vascular SMC-specific ion channels have as yet been identified. There is, however, clear evidence for differential regulation of ion channel function in SMCs (113). This suggests that at least some of the molecules that regulate ion channel function in SMCs may be SMC specific/selective and could be used as differentiation markers, although as yet, no studies have examined the developmental regulation of these factors.

IV. DEVELOPMENTAL ORIGIN OF VASCULAR SMOOTH MUSCLE

Early investigators have provided detailed descriptions of the development of extra- and intraembryonic blood vessels (91, 158, 186, 199, 205). From these studies it is apparent that two separate processes contribute to vessel formation. Vasculogenesis is the de novo formation of blood vessels in which vascular precursor cells, angioblasts, differentiate from mesoderm to form endothelial cells, which then coalesce to form intact blood vessels. Angiogenesis is the process by which the vascular network is extended by the budding of preexisting vessels. In each of these cases, the blood vessel initially consists of a single layer of endothelial cells embedded in a scaffolding of extracellular matrix (173, 205, 210). Development of larger blood vessels, i.e., arteries, arterioles, venules, and veins, from these initial vessels involves recruitment of SMC precursor cells into the region surrounding the endothelial tubes with subsequent morphogenesis of the appropriate blood vessel (see also sect. VII*B1*). While much is known regarding the embryological origins of vascular endothelial cells that line the blood vessels (reviewed in Refs. 48, 186, 205), relatively little is known regarding the origins of the precursor cells that give rise to vascular smooth muscle cells.

Morphological studies have shown that during vasculogenesis "putative" smooth muscle precursor cells appear at stage 11 [Hamburger and Hamilton (102), *day 2* of development] in the developing chick embryo as one to two layers of relatively undifferentiated mesenchymal cells that surround the developing endothelial cells of formed vessels (91, 115, 173, 199). By stage 19 (ca. 68–72 h of development), the cells that surround the endothelial cell layer are recognizably different from undifferentiated mesenchyme but are not yet clearly recognizable as smooth muscle in that they lack basement membranes, attachment bodies, or pinocytotic vessels. However, cells have abundant thin filaments and may already possess contractile activity as indicated by the smaller caliber of the vessels at this stage. After stage 22 (ca. 3.5 days of development), there is continuous accretion of mesenchyme into smooth muscle, and veins and arteries become clearly distinguishable. Arterialization of developing capillaries, i.e., incorporation of SMCs around endothelial cells that form the capillary, coincides with establishment of blood flow and an increase in blood pressure within the endothelial tube (120). This has led to speculation that mechanical forces play an important role in recruitment and differentiation of SMCs during arterialization.

A limitation of early studies was that they relied on morphological characteristics that are not unique to SMCs, as well as juxtaposition to endothelial cells as the sole means for identification of SMCs. This may have led to misidentification of SMCs and prohibited early positive identification of SMC precursors before their recruitment into the vessel wall. A number of investigators have attempted to identify the earliest origins of vascular SMCs by using antibodies directed against proteins that are characteristic of differentiated SMCs. The most extensively studied marker has been smooth muscle α-actin, and this appears to be the earliest known marker of differentiated SMCs expressed by presumptive SMCs that surround the primitive endothelium in the developing dorsal aortas (64, 122). Smooth muscle α-actin is transiently expressed in the epimyocardium of the avian embryo between Hamburger and Hamilton stage 8–10 (221, 260), *Xenopus* from stage 37/38 and stage 46 (222), and the rat at gestational *day 10* (229, 291). Expression then becomes rapidly restricted to visceral SMCs of the presumptive gut and the vascular SMCs of large vessels in the anterior portion of the embryo, where it has been detected as early as Hamburger and Hamilton stage 18 (or *day 2.5* of development) in the quail (64, 122), stage 40/41 in *Xenopus* (222), and gestational *day 10* in the rat (229). In the rat, smooth muscle actins were not observed in developing smooth muscle of other organs until *day 14*, when the mesenchyme condensing around the developing lung and gut stained with smooth muscle α-actin antibodies. Unfortunately, no markers have been described that recognize smooth muscle precursor cells (i.e., committed but undifferentiated SMCs), and no studies have as yet been reported using tagging techniques to map the earliest origins of vascular SMCs. As a consequence, the origins of SMCs are poorly defined.

Gross mapping of the embryological origins of vascular SMCs, however, has been accomplished using interspecies grafting techniques in which regions of developing embryos from one species (usually quail) are surgically

removed and transplanted to a host embryo from another species (150, 205). The species of origin of cells typically is identified based on nucleolar appearance or some other distinguishing trait. Results of these studies have shown that vascular SMCs are derived from the splanchnic layer of the ventrolateral plate mesoderm (150). The exception to this is for the great vessels of the head and neck, which are derived from neural crest mesectodermal cells (118, 150). Note that this exception is by no means unique to SMCs, in that in the head, many of the neural crest cells will differentiate into cartilage, bone, and other connective tissues, which elsewhere in the body arise from the mesoderm.

A major limitation of studies of SMC development is that no studies as yet have defined the origins or more importantly the precise timing of the inductive event that results in commitment of multipotential cells to the SMC lineage. However, given the extreme plasticity of fully differentiated SMCs and their ability to assume many different phenotypes, consideration of commitment or determination for SMCs is likely to be quite different from in many other cell types such as skeletal and cardiac muscle which undergo terminal differentiation to phenotypes that are much more restricted in nature. In any case, there is a need for embryological tagging studies using retroviruses, or other markers to identify the precise origins of vascular smooth muscle and the timing of the inductive event. It has been suggested that SMCs arise at multiple diffuse sites throughout the embryo, rather than from a discrete locus or loci. However, this cannot be definitively ascertained until the precise timing of the inductive event that results in commitment of multipotential cells to the SMC lineage is clearly defined. Although a separate issue, we have recently presented evidence suggesting that arterialization (i.e., the formation of arteries from capillaries) during angiogenesis involves recruitment of SMCs from upstream feed vessels (i.e., terminal arterioles) rather than recruitment of "new SMCs" from primitive precursor cells from within the mesenchyme (206).

V. DEVELOPMENTAL CHANGES IN CONTRACTILE PROTEIN EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS: EVIDENCE FOR ASYNCHRONOUS DIFFERENTIATION

As indicated in sections I–IV, there is clear evidence for developmental regulation of contractile protein expression in vascular SMCs. However, as yet, complete mapping of the time course of changes in expression of each of the known SMC differentiation marker proteins over the entire development time course has not been completed in a single species. In addition, there are some uncertainties regarding the specificity of some of the biochemical techniques and antibodies employed to assess expression of contractile protein isoforms in certain studies. As a consequence, it is difficult to construct a complete chronology of changes in SMC contractile protein expression during development of the vascular system. Nevertheless, certain clear patterns are emerging. These are summarized in Figure 1, which shows the ontogeny of changes in contractile protein expression in vascular smooth muscle in the developing vasculature. The presumption in this diagram is that there is a continuum of differentiated phenotypes that exists between the committed but as yet undifferentiated SMC precursor cell at one end of the spectrum and the fully contractile mature SMC at the other end of the spectrum.

The earliest known marker of differentiated SMCs is smooth muscle α-actin, which is induced coincident with recruitment of presumptive SMC precursors into the vessel wall (64, 166). There is then sequential induction of additional differentiation-specific marker proteins that continues late in vascular development, with SM-1 MHC, calponin, h-caldesmon, and SM-22 being markers of the intermediate stages of SMC differentiation/maturation (64, 122, 144, 164) and SM-2 MHC being a marker of late differentiation or maturation stages (1, 144, 166).

Under normal circumstances, the expression of most, if not all, of the SMC differentiation markers appears to be relatively uniform between SMCs within mature blood vessels (64, 144, 166), with some possible exceptions that will be discussed later in this section. However, this is clearly not the case during development, where there is clear evidence for heterogeneity in contractile protein expression between different SMCs within a given blood vessel. Such observations indicate that the developmental timing of expression of the various SMC differentiation markers is not synchronous (84, 140, 197). For example, whereas the majority of vascular smooth muscle cells in near-term fetuses and newborn rats express smooth muscle α-actin, our studies (197) and those of Kocher and Gabbiani (140) demonstrated that a subpopulation of medial cells, presumably SMCs, in the newborn rat aorta express no detectable smooth muscle α-actin based on staining with a monoclonal antibody specific for smooth muscle α-actin (248). The [^{3}H]thymidine labeling index was much higher in these cells than in smooth muscle α-actin-containing cells, suggesting that there is a subpopulation of relatively undifferentiated SMCs in the newborn rat aorta that show increased proliferation (197). However, consistent with ultrastructural studies showing that mitotic SMCs contain abundant myofilaments (47, 124), some smooth muscle α-actin-positive cells were labeled with [^{3}H]thymidine (197), indicating that differentiation and proliferation are not mutually exclusive in vascular smooth muscle. Similar evidence for heterogeneity of expression of smooth muscle MHC isoforms between different SMCs within a given blood vessel has been observed by Sartore and co-workers (84) in the developing rabbit aorta.

Developmental increases in contractile protein content in smooth muscle correlate closely with increasing contractility of the tissue and are undoubtedly important in this regard (69, 174). However, the functional significance of shifts from nonmuscle to smooth muscle variants of contractile proteins is much less clear, given the high degree of homology between these proteins. Moreover, with few exceptions, there is little direct evidence that

nonmuscle and smooth muscle variants differ significantly with regard to biochemical or biophysical characteristics (see Refs. 253 and 204 for reviews), implying that there may not be major functional differences between isoforms. An alternative possibility is that multiple isoforms of many of the contractile proteins evolved, at least in part, as a means of attaining tissue-specific regulation of proteins that are ubiquitous to all cells. For example, proteins such as actin and myosin must be present to some extent in all cells, since they are required for cytokinesis and cell motility (204). However, the high mechanical forces produced by SMCs require that they express much higher levels of these proteins than nonmuscle cells (174). As such, it is clear that an understanding of the regulation of differentiation of vascular SMCs will be dependent not only on mechanisms that lead to qualitative shifts in which contractile protein isoforms are expressed, but also on what controls the very high level of expression of these proteins in fully differentiated SMCs. It is also possible, however, that there are important functional differences between the nonmuscle and smooth muscle variants of the various contractile proteins that remain to be identified (see sect. III).

Whereas the SMC's principal function in mature tissues is contraction, this cell also has important synthetic functions during development, as well as after vascular injury, and it is the major source of the extracellular matrix components of the blood vessel wall (39, 103, 183, 241). Thus any consideration of the developmental biology of the SMC must take into account this multifunctionality. Moreover, whereas the contractile and synthetic functions of smooth muscle appear to be inversely correlated to some extent, they are clearly not mutually exclusive. Smooth muscle cells in developing blood vessels simultaneously express multiple smooth muscle contractile proteins and a variety of extracellular matrix proteins including elastin and collagen (103, 241). Furthermore, fully differentiated SMCs in mature blood vessels continue to synthesize extracellular matrix components, although at a reduced level, and retain the ability to increase this synthesis following vascular injury (183). Thus there is a continuum of phenotypes available to the SMC ranging from a developmentally immature but committed SMC that proliferates rapidly and expresses low levels of smooth muscle contractile proteins to the fully differentiated SMC that shows a low proliferative rate and is a cell geared almost exclusively to contraction.

There is little evidence that there are subtypes of SMCs in adult animals that subserve primarily a synthetic/secretory role under normal conditions (1, 64, 73, 144, 166). Rather, when such cells appear, such as after vascular injury or in diseases such as atherosclerosis, they appear to be derived from fully contractile SMCs (45, 238). An exception is in chickens in which there is a subtype of medial cell in the normal adult aorta, termed the intralaminar cell, that is clearly distinguishable on a morphological basis (169). This cell is much smaller than normal contractile SMCs and contains an abundance of ribosomes, Golgi apparatus, and other cellular organelles which suggests that it is a highly synthetic/secretory cell. Little is known regarding this cell type, including whether it actually represents a true SMC lineage, and whether an equivalent cell type (although presumably having less overt differences in its morphology, frequency, etc. that has allowed it to escape detection) might also exist in mature blood vessels in mammalian species.

VI. UTILITY OF CULTURED SMOOTH MUSCLE CELLS FOR STUDYING REGULATION OF SMOOTH MUSCLE CELL DIFFERENTIATION

Consideration of the factors and mechanisms that regulate the differentiated state of SMC is complicated by the extreme plasticity of this cell type which enables it to undergo rapid and reversible changes in its phenotype in response to environmental influences. Indeed, maintenance of the differentiated state of the SMC is likely to be dependent on continuous regulation, rather than more permanent regulatory controls, to a much greater extent than cells with less cellular plasticity such as skeletal muscle (22). This, coupled with evidence that SMCs are derived from multiple lineages, suggests that SMC differentiation is under control of complex local cues that at present are not well defined. Whereas mechanical, metabolic, neural, and humoral stimuli are likely to play an important role, relatively little direct evidence is available to demonstrate an effect of these factors on SMC differentiation/maturation, and almost nothing is known regarding the precise mechanisms whereby these stimuli might manifest their effects.

The relative paucity of information regarding regulation of SMC differentiation is due in part to inadequacies in the early SMC cultures that were available for studying SMC differentiation. This included limitations with respect to the inducibility and/or retention of the differentiated phenotype in vitro, to inadequate or incomplete characterization of the differentiated phenotype of the cultured SMCs, and in many instances to the failure to clearly establish that the cells studied were SMCs. An example of the latter is the BC_3H1 cell line which was originally derived from a muscle brain tumor (233) and was purported by a number of investigators to be a model for studying SMC differentiation. However, there is now unequivocal evidence that this is not the case in that this cell expresses almost exclusively skeletal muscle variants of contractile proteins (269) and does not express SMC differentiation markers such as smooth muscle MHC (164; G. Owens and M. McCanna, unpublished data) or calponin (81). Although the cell does express smooth muscle α-actin (259), this alone cannot be interpreted as evidence of SMC lineage, particularly since skeletal muscle in vivo also transiently expresses this gene during development (229, 291). The BC_3H1 cell does, however, provide an interesting system with which to study molecular regulation of smooth muscle α-actin expression in non-SMC systems (71). A number of other purported SMC lines such as A7r5 and A10 (138) express some SMC differentiation markers

such as smooth muscle α-actin, but require characterization of additional markers to unequivocally establish their SMC lineage.

Much progress has been made in recent years to improve the SMC cultures that are available with respect to retention of differentiated properties, as well as the criteria utilized to evaluate their identity and differentiated state (32, 132, 140, 176, 193, 215, 216). As such, it has only been in recent years that acceptable culture models have become available with which to begin to systematically investigate regulation of SMC differentiation.

Smooth muscle cells, like virtually all cultured cells, undergo extensive changes in their differentiated phenotype when grown in vitro (32, 40, 82, 132, 140, 193, 215, 216, 239, 240). This is not surprising given the plasticity of the SMCs and the fact that it is virtually impossible to mimic in vivo conditions in culture. The so-called phenotypic modulation process was first described in a series of pioneering studies by Chamley-Campbell et al. (see Ref. 40 for a review), who found that vascular SMCs showed gradual loss of morphologically and immunocytologically identifiable myofilaments as well as contractility when grown in culture. Consistent with these results, it was subsequently demonstrated that SMCs show decreased expression of a number of smooth muscle contractile proteins including smooth muscle α-actin (32, 193), smooth muscle MHC (132, 216), calponin (82, 241), SM-22α (82, 241), h-caldesmon (82), vinculin/metavinculin (12), and 20-kDa myosin light chains (168, 268), as well as increased expression of the nonmuscle variants of these proteins when placed in culture. Importantly, although we (193, 216), like Chamley-Campbell et al. (40) found that alterations in actin and myosin content occurred relatively slowly when SMCs were placed in culture, changes in the synthesis of these contractile proteins occurred immediately. This indicates that the SMC rapidly alters its gene expression patterns in response to cell isolation procedure and placement in culture and that the slow kinetics of changes in contractile protein content are likely a function of the relatively long half-lives of these proteins. The rapidity of the changes in gene expression that occur when SMCs are isolated is of critical importance when considering possible uses of either freshly isolated or cultured SMCs. This is particularly true if the experimental questions being addressed involve relatively short-lived regulatory molecules.

The extent of phenotypic modulation and its reversibility appears to be dependent on many factors, and this has undoubtedly contributed to many of the controversies that exist in the literature regarding the biology of the vascular SMC. Critical factors include the methods of cell isolation and cell passaging, the initial plating density, the presence or absence of serum or mitogens, the specific lot of serum used, the age of the animal from which cells are derived, and the substrate on which cells are grown (21, 40, 51, 111, 176, 239, 270, 271). It is now clear that there is not complete and irreversible loss of smooth muscle-specific contractile proteins in cultured SMCs. Numerous investigators have shown that SMCs derived from a variety of blood vessels and species can be grown under conditions in which they continue to express smooth muscle contractile proteins (32, 40, 43, 132, 140, 193, 215, 216, 239) as well as contractile responsiveness (24, 168, 176, 262) for many passages, if not indefinitely in culture. For example, the rat aortic SMCs used extensively in our laboratory undergo agonist-induced Ca^{2+} transients and MLC phosphorylation in response to a variety of different contractile agonists including angiotensin II, norepinephrine, endothelin, and arginine vasopressin (168). These SMCs also continue to express all of the known SMC differentiation markers thus far examined including smooth muscle α-actin, smooth muscle MHCs, h-caldesmon, smooth muscle α-tropomyosin, and smooth muscle MLC, albeit at reduced levels relative to expression of the nonsmooth muscle variants of these proteins (116, 168, 193, 216). We have found that a key to retaining expression of these differentiation markers is to optimize for the initial yield of viable cells, and to passage cells at subconfluency so as to avoid selection for cells that show loss of contact inhibition of growth. Other investigators have reported methods whereby SMCs retain contractile capabilities through multiple passages in cell culture (24, 176). In most of these cases, SMC contraction was assessed on cells grown on deformable substrates rather than directly on nondeformable plastic culture dishes, thus permitting cell shortening without cells having to disengage from the underlying matrix. Indeed, in many cases, the ability to show contractile ability in cultured SMCs may be due to a detection problem rather than inherent differences in contractile capability of SMC primary lines derived using different cell-culture methods. However, this is not to undermine the critical importance of culture methodologies in determining the extent to which cultured SMCs express characteristics of differentiated SMCs in vivo, a topic that is considered in further detail below. Before reviewing this area, however, it is important to first consider why we use cultured cells, their principal limitations, and what are appropriate versus inappropriate questions to address using them.

The two principal advantages of using cultured cells are *1*) to allow better control of experimental variables, that is, identical replicate plates of a single cell type can be exposed to a single experimental variable, something which is almost never possible in intact tissue or whole animal experiments due to genetic diversity between animals, cell-cell interactions, indirect or unknown effects of your experimental test variable, etc.; and *2*) to provide a more reliable, manipulatable, and/or consistent source of relatively large amounts of biological material that is often needed for biochemical or molecular studies. The major disadvantage is that cells are undoubtedly modified as compared with their in vivo counterparts. As such, one must be careful as to what types of questions are asked and appreciate that any results obtained may not necessarily reflect what occurs in vivo in fully differentiated SMCs in their normal environment. Nevertheless, cultured cell systems provide a very powerful means to study key regulatory pathways that are of interest based on in vivo

studies and, in particular, a means to identify the key components and regulatory controls in that pathway that would not be possible with any other experimental approach. This information can then be tested in a variety of in vivo animal model systems including use, at least in the mouse. of rapidly improving technologies for making transgenic and/or gene knockout/replacement animals (see reviews in Refs. 8, 98, 211).

A very common practice in the vascular biology field has been to try to elucidate possible in vivo differences between vascular SMCs on the basis of comparison of the properties of cultured cells derived from different animal models or different vascular sources (e.g., intima vs. medial cells). Whereas this has yielded some useful information, results obtained with such an approach must be viewed with extreme caution. A major problem is that there is clear evidence that SMCs derived from a different original source, including a different species, different strains of the same species, different ages of animals of the same strain, or even different blood vessels within an individual animal, can undergo differential phenotypic modulation under identical culture conditions; that is, differences that existed in vivo result in the cells responding differently to a given set of culture conditions. This problem is illustrated by the following example, which is just one of many of its type that exist in the SMC field.

Numerous studies have attempted to elucidate mechanisms that are responsible for the altered growth of vascular SMCs that occurs in vivo in association with hypertension, on the basis of comparison of the growth properties of SMCs derived from blood vessels of hypertensive versus normotensive animals. The most common example has involved direct comparison of the growth properties of cultured SMCs derived from the spontaneously hypertensive rat (SHR) as compared with those derived from its normotensive counterpart the Wistar-Kyoto rat (WKY). In addition to the problems relating to the genetic diversity between these two rat strains that compromises even in vivo studies (reviewed in Ref. 255), there is clear evidence that aortic SMCs from these two different rat strains undergo differential phenotypic modulation under identical conditions of cell culture. For example, we (G. Owens and A. Geisterfer, unpublished data) and Rosen et al. (212) found that SMC derived from WKY, but not those derived from the SHR, reverted to a polyploid state when grown in in vitro culture. This is just the opposite of what occurred in vivo, where the SHR SMCs preferentially developed polyploidy (196). The cultured SMCs derived from SHR grow faster than those derived from WKY (198, 223), although differences in proliferation rates are not observed in vivo (194, 196). Rather, medial hypertrophy of the aorta in SHR is due to enlargement or hypertrophy of existing SMCs with little or no SMC proliferation. As such, it is clear that any direct comparison of growth properties or any other parameter between SMC derived from these two species involves a large number of undefined variables relating to the differential effects of culture on the two cell types. This is just one of many examples that illustrate the potential problems associated with attempting to deduce in vivo differences on the basis of direct comparisons of the properties of SMCs derived from original sources that differ in any significant way.

This is not to say that such an experimental approach cannot be a productive means of investigation. Indeed, studies from a number of laboratories have identified potentially interesting SMC genes on the basis of qualitative comparisons between SMCs derived from different sources. For example, Schwartz and co-workers (80, 151, 235) and others (103, 241) have identified a number of genes that show altered expression during development, including osteopoitin, elastin, insulin-like growth factor II, F-31, and SM-22α by comparing gene expression patterns between cultured aortic SMCs derived from newborn versus adult rats. Similarly, several genes that show altered expression in intimal lesions of atherosclerotic plaques were first identified based on differential expression in cultured SMC derived from intimal versus medial blood vessel segments (283). Results of these studies have shown that SMCs derived from different sources show markedly different phenotypes in culture. Importantly, however, the phenotypes exhibited are remarkably consistent between different independent primary cultures from a given source, and remarkably stable for many passages in culture. An example are the "pup"- versus the "adult"-derived SMC cultures described by Schwartz and co-workers (80, 151, 235). The adult SMCs, which are derived from aortas of 3- to 5-mo-old Sprague-Dawley rats, show the typical hill-and-valley morphology that is characteristic of most SMC cultures, and their growth is dependent on exogenous growth factors. In contrast, the pup-derived SMC, which are derived from aorta of 3- to 4-day-old Sprague-Dawley rats, show an "endothelial"-like cobblestone morphology and are capable of growing in the absence of exogenous growth factors (235). Interestingly, the pup-derived cells exhibit a number of features in common with cells derived from intimal lesions of adult animals (283). The reasons for these strikingly different phenotypes are not clear, although at least some of the differences observed could represent differential phenotypic modulation of cells derived from different sources to the particular culture conditions employed. However, some of the differences in gene expression found in cultured SMCs derived from different sources, such as alterations in elastin, osteopontin, and SM-22α expression (80, 103, 241), have also been found to exist in vivo. As such, use of these SMC culture systems may be valuable in identifying the cellular and molecular processes that control some of the many "alternative phenotypes" of the vascular SMC. However, it is critical that evidence be provided that the "phenotype" observed is biologically relevant, and not simply an artifact of in vitro culture.

A wide variety of different methods have been described for isolating and culturing vascular SMCs derived from multiple blood vessels and species of origin. These have previously been reviewed by Chamley-Campbell et al. (40) and will not be discussed in detail here. Two general methods are employed for initially obtaining the cells: *1*) enzymatic dissociation into single cells using vari-

ous proteolytic enzymes including elastase, collagenase, dispase, and trypsin or *2*) explant techniques in which cells migrate out of small pieces of tissue. Although no doubt an oversimplification, the general experience has been that SMCs derived by enzymatic dissociation retain their differentiated properties to a greater extent than do explant-derived cells. This may relate to the additional selection pressure on these cells in that they must be capable of migrating out of the tissue segment to be cultured. The explant-derived SMCs, however, may provide a model system that better mimics the pathological state of the SMCs exhibited within intimal lesions. Smooth muscle cell cultures maintained in the absence of serum or other purified mitogens have been reported to show delayed phenotypic modulation (247, 262). Whereas this is of use for certain types of short-term culture experiments, it obviates one of the principal reasons for using cultured cells, i.e., to produce large amounts of biological material. Moreover, as discussed in section VII*A*, it is clear that proliferation and retention of many differentiated properties in SMCs are not necessarily mutually exclusive.

As is evident from the preceding, there is no right or wrong culture method to use. Rather, the choice should be dependent on what experimental question is being addressed. As a starting point, the investigator is advised to adopt a method already in the literature which appears to best retain the specific properties of interest. The cell culture system selected then needs to be carefully characterized for the properties of interest in that laboratory to test for differences that are likely to occur due to the use of different isolation enzymes or serum lots.

VII. FACTORS THAT INFLUENCE THE DIFFERENTIATED STATE OF VASCULAR SMOOTH MUSCLE CELLS

A. Growth State

While it is clear that SMCs in culture are phenotypically modulated and show much increased rates of growth as compared with their in vivo counterparts, the relationship between growth and differentiation in vascular SMCs is not fully understood. It has been suggested, based on observations that loss of myosin- and actin-containing filaments preceded onset of cell proliferation in primary culture, that SMCs normally exist in a nonproliferating "contractile state" and must modulate to a "synthetic state" as a prerequisite for cellular proliferation (40, 270). Whereas this is an attractive hypothesis, there are a number of limitations in the experimental data supporting it. A reoccurring weakness has been the inability to distinguish between changes that were truly growth related and those that merely represented an adaptation to growth/survival in culture. In addition, data were strictly correlative in nature, and it is unclear whether there was a cause-effect relationship between alterations in SMC phenotype and onset of proliferation. Other groups (15, 97, 108, 237), including our own (193), do not observe a prolonged lag period (5–7 days) before onset of cell proliferation in culture, and such lag times have not been observed in organ culture systems in which cells are not enzymatically dissociated (58, 117). Furthermore, De Mey et al. (58) showed that loss of contractility in vascular smooth muscle organ culture occurred independent of onset of cell proliferation. Most importantly, the time course for SMC modulation proposed based on experiments in primary cultures of SMCs (40) is inconsistent with the kinetics of initiation of SMC proliferation in vivo following vascular injury (44). This raises the possibility that the delayed onset of proliferation observed may have been due, at least in part, to cell damage during the isolation procedure rather than to a requirement for phenotypic modulation. Consistent with this, electron microscopic studies have clearly shown that mitotic SMCs in intact smooth muscle tissues in vivo express many characteristics of differentiated SMCs (47, 124). Thus, whereas the hypothesis that the differentiated state of a SMC is a critical determinant of its growth responsiveness is attractive, and is supported by an abundance of circumstantial evidence, direct evidence is lacking. Among the central issues that need to be addressed are the following: *1*) What extent of SMC differentiation/maturation is compatible with proliferation? *2*) How distinctive are the various differentiated states that are available to the SMCs and what changes in growth potential are associated with each state? *3*) How reversible are transitions from one state to the other? *4*) What controls these transitions?

Studies in my laboratory, and others, have demonstrated that the effect of growth stimulation per se on expression of smooth muscle differentiation markers is variable depending on the particular means of stimulating SMC growth and the SMC differentiation marker examined (19, 51, 116, 216). For example, in early studies we observed that the fractional expression of smooth muscle isoforms of actin (21, 193) relative to the nonmuscle forms was markedly reduced at the protein level in logarithmically growing subconfluent SMCs as compared with postconfluent contact-inhibited SMCs [both in 10% fetal bovine serum (FBS)] or subconfluent SMCs growth arrested in a defined serum-free medium. However, subsequent studies showed that this was due primarily to growth-induced increases in expression of nonmuscle β-actin rather than a reduction in the expression of smooth muscle α-actin (19, 51). Indeed, the accumulation of smooth muscle α-actin in postconfluent SMCs was not accompanied by corresponding increases in smooth muscle α-actin mRNA levels or synthesis per cell, indicating that the smooth muscle α-actin gene is constitutively active under these conditions and that the accumulation of α-actin in postconfluent cells was mediated posttranslationally by an increased effective half-life of the protein (50, 51). In contrast, synthesis of smooth muscle myosin isoforms SM-1 and SM-2 was increased in growth-arrested cells as compared with growing cells (116, 216). Results indicate that SMCs, unlike cardiac or skeletal muscle cells (188), are capable of sustained expression of at least some muscle-specific contractile proteins during cellular proliferation.

To further explore the relationship between SMC growth and differentiation in SMCs, we compared the effects of platelet-derived growth factor (PDGF)-induced (a mixture of homodimeric and heterodimeric forms derived from human platelets) or serum-induced growth on actin expression in postconfluent quiescent cultures maintained in a defined serum-free medium (19, 50, 51). Whereas both factors elicited a potent proliferative response and decreased fractional smooth muscle α-actin synthesis, their effects on actin isoform expression were quite different. Platelet-derived growth factor induced a rapid drop in smooth muscle α-actin steady-state mRNA level, as well as in the absolute rate of synthesis of smooth muscle α-actin, but had no effect on either nonmuscle β-actin mRNA levels or synthesis (50, 51). In contrast, serum stimulated a marked increase in nonmuscle β-actin mRNA levels and synthesis but had no effect on smooth muscle α-actin expression. Additional studies demonstrated that chronic PDGF treatment was capable of repressing smooth muscle α-actin expression in the absence of sustained mitogenesis, that PDGF-induced repression of smooth muscle α-actin expression was fully reversible, that the A chain of PDGF was not required for these effects, and that the 50% effective dose for PDGF-induced mitogenesis was two- to fourfold higher than that for repression of smooth muscle α-actin expression (19). Other purified growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor, insulin-like growth factor, or combinations of these had little or no effect on either smooth muscle α-actin expression or SMC growth (19). In more recent studies, we found that the effects of PDGF were not limited to smooth muscle α-actin, but rather appeared to represent a more generalized suppressive effect on the expression of a variety of smooth muscle differentiation markers including smooth muscle MHC and smooth muscle α-tropomyosin, which both show rapid and dramatic decreases in expression at both the protein and mRNA levels following PDGF B chain (PDGF-BB) stimulation (116). These effects, like those on actin, were highly selective in that PDGF-BB nearly abolished expression of the smooth muscle variants of these proteins without affecting the nonmuscle variants. In contrast to the effects of PDGF-BB, 10% FBS, which stimulated a mitogenic response equal to or greater than that of PDGF-BB, increased, rather than decreased, smooth muscle α-tropomyosin expression and induced only very modest decreases in expression of smooth muscle MHCs. The failure of serum to effectively repress expression of these smooth muscle differentiation markers did not appear to be due to some inhibitor present in FBS in that high concentrations of FBS failed to block the differentiation-suppressing effects of PDGF-BB. Nevertheless, because serum contains a large number of undefined components that might complicate interpretation of these experiments, we sought to compare effects PDGF-BB with another purified growth factor that has similar efficacy in stimulating SMC mitogenesis. To this end, we have recently shown that thrombin, which induces a mitogenic response equivalent to that of PDGF-BB in our rat aortic SMCs (163), stimulated increased, not decreased, expression of both smooth muscle α-actin and smooth muscle MHC (279).

Taken together, the preceding results indicate *1*) that a drop in expression of a number of SMC differentiation marker proteins is clearly not obligatory for cell cycle entry in cultured vascular SMCs, *2*) that the level of expression of smooth muscle differentiation proteins is not necessarily a direct function of the proliferative activity of the cell but rather appears to be dependent on the specific means of growth stimulation, and *3*) that PDGF-BB is unique among the SMC mitogens tested thus far in its ability to selectively suppress the expression of multiple SMC differentiation markers. Our results, and those of Thyberg et al. (271), suggest that PDGF-BB may play an important role in control of SMC differentiation that may be distinct from its role as a SMC mitogen.

Platelet-derived growth factor B chain-induced decreases in SM actin protein and mRNA expression were not associated with decreased transcription of the smooth muscle α-actin gene, as measured using transcription run-on analyses (50). Rather, PDGF-BB induced a selective decrease in the stability of the smooth muscle α-actin mRNA without affecting the stability of the nonmuscle β-actin mRNA. Platelet-derived growth factor-induced decreases in smooth muscle α-tropomyosin and smooth muscle MHC expression also appeared to be due at least in part to mRNA destabilization, based on observations that the rate of decline in these transcripts following PDGF treatment was more rapid than predicted based solely on cessation of transcription of these genes (116). Indeed, as yet, there is no published evidence showing regulation of the expression of any of the SMC differentiation marker genes in cultured SMCs at the transcriptional level, although granted, very few studies of this nature have been completed. At least some of the genes such as smooth muscle α-actin, smooth muscle α-tropomyosin, and smooth muscle MHC appear to be constitutively on, at least in our cultured rat aortic SMCs, and subject to posttranscriptional regulatory controls (50, 116).

B. Role of Cell-Cell Interactions

1. Endothelial-smooth muscle cell interactions

As discussed previously in section IV, vasculogenesis is initiated by formation of capillaries consisting of a single layer of endothelial cells (186, 205). During arterialization, presumptive SMCs are recruited from the mesenchyme, surround the endothelium, and subsequently differentiate into smooth muscle. It is thus reasonable to suggest that endothelial cells might play an important role in the arterialization process. Indeed, cultured endothelial cells have been shown to secrete a variety of growth factors for smooth muscle, including PDGF and bFGF (49, 78, 104, 127, 209), as well as growth inhibitors such as heparin and TGF-β (31, 36, 228), which can either stimu-

late or inhibit growth of cultured SMCs depending on the experimental conditions (4, 192, 257). Many of these endothelial-derived factors also have chemotactic activity for SMCs and may play a role in recruitment and migration of SMCs or SMC precursors from primitive mesenchyme during vasculogenesis (49, 104, 127).

Endothelial cells may also play a role in control of SMC differentiation. Campbell and Campbell (31) reported that endothelial cell conditioned medium, or coculture of primary cultures of SMCs with confluent but not subconfluent endothelial cells, inhibited growth and prevented or delayed at least some of the phenotypic changes that occur in SMCs when placed in culture. Addition of heparin to the culture media had similar effects, raising the possibility that effects may be mediated by heparin or heparin-like compounds. Both cultured endothelial cells and SMCs are known to secret a heparin-like molecule that inhibits SMC growth (36, 75), with that isolated from postconfluent cells being much more potent than that from subconfluent cells in inhibiting SMC growth. Heparin has also been shown to inhibit myointimal proliferation of SMCs in vivo following vascular injury (42, 43), although it did not prevent injury-induced decreases in smooth muscle α-actin expression. Heparin has been reported to increase smooth muscle α-actin expression in cultured SMCs, but only under conditions in which it had an antiproliferative effect (59). These results suggest that the effects of heparin on SMC differentiation may simply be secondary to growth inhibition rather than a direct effect on SMC differentiation per se.

In contrast to these findings, other investigators have reported that endothelial cell conditioned media stimulated rather than inhibited SMC growth (236). Consistent with these observations, we recently demonstrated that conditioned media from rat aortic endothelial cells stimulated rather than inhibited SMC growth (280). Moreover, we found that endothelial cell conditioned media nearly completely suppressed expression of smooth muscle α-actin, smooth muscle MHCs, and smooth muscle α-tropomyosin at both the protein and mRNA levels. Effects of endothelial conditioned media were highly selective in that no effects on expression of the nonsmooth muscle variants of these proteins were observed. This activity was not inhibitable with neutralizing antibodies to PDGF or bFGF, was heat and protease sensitive, bound with weak affinity to heparin Sepharose (elution at 0.3–0.4 M NaCl), and had an estimated molecular size of 45 kDa based on gel filtration chromatography. Treatment of SMCs with the known endothelial cell factors bFGF, endothelin, TGF-β, vascular endothelial growth factor, or PDGF A chain did not mimic the effects of endothelial conditioned media, suggesting that these factors are not responsible for the SMC differentiation-suppressing activity. Taken together, results indicate that rat aortic endothelial cells in culture secrete an as yet unidentified mitogen that is a potent suppressor of SMC differentiation.

There is thus evidence that endothelial cells can secrete both positive and negative differentiation factors for SMCs. However, relatively little is known regarding what regulates the balance between these opposing activities. It has been shown that endothelial cells in culture produce much higher levels of growth factors than do their in vivo counterparts (10). As such, the predominance of mitogenic and differentiation-suppressing activity in our endothelial cell cultures may well reflect the properties of endothelial cells that are relatively poorly differentiated. Key questions to be addressed include the following: Which endothelial cells-derived factors regulate the differentiation of vascular SMCs? What regulates endothelial cell production of both positive and negative SMC differentiation factors? Do SMCs influence the differentiated state of endothelial cells?

2. *Neuronal influences*

Interruption of neuronal input has been shown to result in decreased growth and loss of contractility in various smooth muscle tissues (16), thus implicating a role for the nervous system in regulation of growth and differentiation of SMCs. Chamley and co-workers (38, 40) reported that the presence of extrinsic sympathetic neurons delayed phenotypic modulation and growth in primary cultures of pig vas deferens and rabbit aorta and ear artery. Conditioned media from nerve cells did not have similar effects, suggesting that the factors produced are labile or that physical proximity is important for the observed effect. These studies involved morphological assessment of the differentiated state of SMCs on the basis of the relative abundance of actin filaments, and no further studies have been reported in this important area using more definitive indexes to assess the differentiated state of the SMCs.

There is also evidence that the SMCs play a role in development of neuronal innervation. Ablation of smooth muscle targets has been shown to inhibit innervation of that tissue, and both cultured SMCs as well as SMCs in vivo have been shown to produce nerve growth factor (274).

C. *Role of Hemodynamic and/or Mechanical Factors*

There is considerable circumstantial evidence that mechanical factors or hemodynamic forces such as shear stress and tangential wall stress may play an important role in development of the vascular system (83, 120, 254). Girard (83) found that incorporation of SMCs into developing avian arteries coincided with establishment of blood flow and an increase in luminal hydrostatic pressure. Hu and Clark (120) characterized the hemodynamic forces in stage 12 through stage 29 chicken embryos and found that systolic blood pressure increased from 0.32 Torr at stage 12 to 2.0 Torr at stage 29 in the chick embryo, during which time there are marked increases in expression of many SMC differentiation marker proteins (Fig. 1). In adult animals, cessation of blood flow and a decrease in hydrostatic pressure are associated with vessel atrophy

and/or remodeling. For example, Langille (145) demonstrated that experimentally induced decreases in blood flow to the carotid artery altered developmental growth in immature animals and caused extensive vessel remodeling in mature animals through an endothelial cell-dependent process. There is extensive evidence that mechanical stress/strain plays an important role in the regulation of growth and maturation of skeletal muscle (for reviews, see Refs. 246, 277). Mechanical stretching of cultured SMCs has a variety of effects including inducing reorientation of cells (56, 130), increased protein and DNA synthesis (288), and increased production of extracellular matrix components (153). Of particular interest, Kanda et al. (130) found that cyclic stretching of bovine aortic SMCs within a three-dimensional matrix of type 1 collagen-induced reorientation of cells parallel to the direction of stretch and increased the relative abundance of myofilaments and dense bodies as compared with nonstretched cultures. In contrast, Dartsch et al. (56) found that cyclic stretching of SMC monolayer cultures resulted in reorientation of cells nearly perpendicular to the direction of stretch. Whereas results of these studies suggest that mechanical factors play a role in vascular growth and development, the precise stimuli that are important for these effects and the signal transduction pathways involved are not known. Moreover, no studies to my knowledge have directly investigated the effects of mechanical stimuli on SMC differentiation based on quantitative assessment of expression of smooth muscle differentiation marker proteins. However, we have demonstrated that contractile agonists such as angiotensin II and arginine vasopressin induce selective increases in the expression of smooth muscle α-actin and smooth muscle MHC in cultured SMCs (273). Because these agonists also stimulate increased MLC phosphorylation and shape changes consistent with cell contraction (168; Owens and Geisterfer, unpublished data), it is possible that the effects observed are secondary to active stress development.

D. Role of Extracellular Matrix

The extracellular matrix has profound effects on cell behavior, including cell migration, proliferation, and differentiation, and undoubtedly plays a key role in vasculogenesis (see Refs. 210 and 33 for reviews). To date, much of the characterization of the role of the extracellular matrix in vasculogenesis has been carried out in studies examining early tubular heart development. Drake and Jacobson (61) observed that the area encompassed by the primitive heart extracellular matrix coincided directly with the boundary of endocardial vasculogenesis. Viragh et al. (281) suggested that the ventral mesocardium and its associated extracellular matrix are important in providing directional cues to the primordial endothelial cells that form the endothelial tubes of the heart. Work from many laboratories has shown the presence of various extracellular matrix components in vasculogenic regions (33, 155). Three-dimensional matrices containing collagen and laminin have been shown to promote formation of tubule structures resembling capillaries in cultured endothelial cells (95). No information is available on whether SMCs associate with endothelium under tube-forming conditions or whether differentiation of SMCs is promoted under these conditions.

Cultured SMCs produce a variety of extracellular matrix components including various collagens, elastins, laminin, fibronectin, and glycosaminoglycans (33). Smooth muscle cells also express integrins that serve as binding sites to the extracellular matrix. Growth of cultured SMCs on various extracellular matrices such as collagen, Matrigel (a basement membrane-rich matrix material isolated from EHS tumor cells), laminin, and fibronectin (46, 8[illegible] 111, 203) has been shown to evoke changes in SMC morphology consistent with a change in the differentiated phenotype of the cell. However, relatively few studies, as yet, have examined the effects on expression of specific SMC differentiation marker genes. Hedin et al. (111) found that growth of cultured SMCs on fibronectin resulted in decreased expression of smooth muscle α-actin. We (Thompson and Owens, unpublished data), as did Pauly et al. (203), found that SMCs grown on Matrigel exhibited a much more spindlelike morphology and a greater density of actin filaments than did SMCs grown on standard tissue-culture plastic. However, we found no changes in the expression of smooth muscle α-actin or smooth muscle MHC under these conditions. We do not feel that these results can be interpreted as evidence that these factors are not important in control of SMC differentiation. Rather, we believe the results reflect limitations of the in vitro culture system and/or the specific experimental conditions and matrices examined. Clearly, further studies are needed in this important area.

VIII. MOLECULAR REGULATION OF SMOOTH MUSCLE CELL DIFFERENTIATION

A. Transcriptional Control of Smooth Muscle Cell Differentiation

As discussed in sections II–IV, a key to understanding SMC differentiation is to identify factors that regulate the coordinate expression of the family of genes that result in the distinct phenotype of the vascular SMC. It is well established that expression of skeletal, cardiac, and nonmuscle contractile protein genes is transcriptionally regulated with respect to both developmentally timed and tissue-specific expression (reviewed in Refs. 189, 285). Furthermore, a number of "master control genes" that regulate skeletal muscle differentiation or lineage determination, including MyoD, have been identified. These factors have been shown to activate the expression of genes characteristic of mature skeletal muscle cells by functioning as transcription factors that bind to a specific DNA-binding motif, referred to as an E box, that is found in the 5′-flanking regions of a number of skeletal muscle-

specific genes including skeletal α-actin, MLC, MHC, troponin, and tropomyosin. Whereas it is likely that the molecular mechanisms involved in SMC differentiation rely on analogous transcriptional regulatory systems, no SMC-specific regulatory/transcription factors have as yet been identified.

Relatively little is known regarding the molecular mechanisms that control transcription of any of the SMC differentiation-specific genes. Furthermore, at this time, there are a limited number of candidate genes that have been identified that are appropriate for studying transcriptional control of SMC differentiation. Whereas expression of the smooth muscle isoforms of MLC, caldesmon, vinculin, and metavinculin are selective for SMCs and clearly show developmental regulation, they are produced by alternative splicing of genes that are expressed in many cell types (29, 106, 121, 167, 185, 252, 264, 287). Thus these genes are not likely to be suitable models to study SMC-specific transcriptional regulatory systems, although they do provide valuable systems for studying tissue-specific gene splicing. The smooth muscle MHC gene shows perhaps the highest degree of SMC selectivity of any of the SMC differentiation genes identified thus far (1, 73, 74, 87, 144. 164, 216). However, sequence information on its promoter has not yet been reported. Furthermore, characterization of the regulation of this promoter may prove difficult due to the fact that expression of this gene is lost relatively quickly in some SMC cultures, although we have found that it persists even at high passages in our primary rat aortic SMC lines (116). Calponin (i.e., the α- or β-isoforms for avian species or h_1- or l-isoforms for mammals) is another potential candidate gene. However, like smooth muscle MHC, no studies have been reported examining its transcriptional regulation.

The most extensively studied smooth muscle gene is smooth muscle α-actin. Smooth muscle α-actin is the most abundant protein in differentiated SMCs, is required for the high force-developing capabilities of the vascular SMCs, and although transiently expressed in cardiac and skeletal muscle during development and in myofibroblasts during wound healing and in tumors, is exclusively expressed in SMCs and SMC-related cells in the normal adult animal (see sect. IIIA1). Additionally, the expression of smooth muscle α-actin is modulated in SMCs within atherosclerotic lesions and may provide an important index for assessing changes in the phenotypic state of SMCs that are important in the atherogenic process (139, 141, 170).

Schwartz and co-workers (34, 35) characterized several sequences in the 5′-flanking DNA of the chicken smooth muscle α-actin gene that regulate expression of a linked structural CAT gene when transfected into embryonic chick "fibroblasts" of undefined origin. They described a 122-bp "core" promoter that was capable of directing constitutive CAT expression in embryonic chick fibroblasts as well as in skeletal myotubes. Immediately upstream from the core promoter was a 29-bp element (−151 to −123), designated a CCAAT-associated repeat or "CBAR," which restricted core promoter activity and was in turn modulated by a 107-bp "governor" sequence (−151 to −257) which was sufficient to suppress the core promoter in skeletal myotubes. Whereas these studies provided important information regarding control of smooth muscle α-actin gene expression in non-SMCs, they did not examine control mechanisms in SMCs. Results of our recent studies done in collaboration with R. Schwartz using chicken smooth muscle α-actin promoter/CAT constructs demonstrated that a quite different mode of regulation occurred in SMCs and in nonmuscle cells derived from adult tissues (18). The core promoter described by Carroll et al. (35) had no activity in nonmuscle cells and low activity in cultured rat aortic SMCs. In addition, the 29-bp region upstream of this core promoter, that acted as a potent negative control element in embryonic fibroblasts and skeletal myoblasts/myotubes, acted as a positive element in smooth muscle, as did further upstream regions between −151 and −257. Thus regulation of expression of the smooth muscle α-actin gene, like other actin genes, appears to be extremely complex and shows quite distinct patterns of regulation in different cell types.

Further evidence for cell type-specific regulation of the smooth muscle α-actin gene has been shown in more recent studies of this gene in both our laboratory (243) and others (71, 256). Studies of the mouse smooth muscle α-actin promoter transfected into mouse AKR-2B fibroblasts and subconfluent mouse BC_3H1 skeletal myoblasts demonstrated that the region between −224 and −192 abolished expression of the first 191 bp of the promoter in both cell types (71, 165). However, site-directed mutagenesis showed that the negative-acting *cis*-element within the −224 to −192 region that abolished activity in BC_3H1 cells was different from the negative element in fibroblasts.

We have recently completed studies characterizing *cis*- and *trans*-acting sequences that confer cell type-specific expression of the rat smooth muscle α-actin gene promoter (243) that was originally cloned in this laboratory (18). These studies involved mapping control elements of the gene by transient transfection of various deletion and site-directed mutants of the smooth muscle α-actin promoter coupled to a CAT reporter gene into SMCs as well as non-SMCs including rat aortic endothelial cells and L6 skeletal myoblasts and myotubes. Smooth muscle cells and skeletal myotubes express the endogenous smooth muscle α-actin gene, whereas endothelial cells and skeletal myoblasts do not. Results provided clear evidence of cell type-specific regulation of the smooth muscle α-actin gene promoter. For example, whereas a truncated promoter consisting of the first 125 bp of the promoter had high activity in SMCs and endothelial cells, this construct had little or no activity in skeletal myoblasts or myotubes. In contrast, promoter constructs containing 547 bp (−1 to −547) or more of the promoter were not expressed in endothelial cells or myoblasts but were expressed at high levels in SMCs and skeletal myotubes, cell types that express high levels of the endogenous smooth muscle α-actin gene. In skeletal muscle, but not in smooth muscle, high transcriptional activity of the promoter was

dependent on inclusion of a region from −125 to −270 that contained two highly conserved E boxes.

The first 125 bp of the smooth muscle α-actin promoter contain two CARG box elements, which have the general sequence motif of $CC(A/T)_6GG$, that are 100% conserved between the four species in which the smooth muscle α-actin promoter has been cloned, suggesting that these elements may have an important regulatory role. The 5′-flanking regions of both the skeletal and cardiac α-actin genes contain conserved CARG box elements that have been shown to direct developmental and tissue-specific expression of these genes, and CARG elements have also been implicated in the regulation of the MLC, muscle creatine kinase, and dystrophin genes in skeletal muscle (reviewed in Ref. 227). The CARG motif was first identified as the core component of the serum response element, a DNA element that is required for the transient transcriptional response of may nonmuscle-specific early-response genes, such a c-*fos* and β-nonmuscle actin, upon serum or growth factor stimulation (114). Although previous studies have shown that the smooth muscle α-actin CARG elements can function in serum-inducible expression of the gene in fibroblasts (71, 137), neither the endogenous smooth muscle α-actin gene nor transfected smooth muscle α-actin promoter-CAT reporter gene constructs are serum inducible in SMCs, even though the nonmuscle β-actin expression is serum stimulated under the same conditions (51, 243).

We demonstrated that mutation of the two proximal CARG boxes [i.e., $CC(AT)_6GG$] located at −62 (CARG A) or at −112 (CARG B) completely abolished the activity of a 125-bp promoter construct in transient transfection assays in SMCs but not in endothelial cells, indicating that these elements are involved in cell type-specific regulation of this promoter (243). Electrophoretic mobility shift assays demonstrated binding of a smooth muscle nuclear protein or protein complex to the CARG elements, whereas similar factors/complexes were not present in non-SMC nuclear extracts. At least one of the SMC CARG-binding factors was serum response factor (SRF) or an SRF-like protein, since SRF antibodies retarded the migration of the SMC complex, and cross-linking experiments demonstrated binding of a 67-kDa protein (the molecular mass of SRF) to the CARG element. Whereas SRF is certainly not a SMC-specific transcription factor, studies of CARG and SRF-mediated transcriptional control in other promoters have shown that specificity of regulation via this element is conferred by a combination of differences in the DNA sequences that flank the CARG element, and by SRF-binding proteins (also referred to as SRF accessory proteins or SAPs) that regulate the function of the SRF in a cell type- and promoter-specific fashion (99, 156, 159, 224, 227). As such, SMC-specific SRF accessory proteins, or regulation of such proteins, may play a key role in SMC type-specific regulation of this gene. In this regard, it is of interest that tissue-specific expression of the skeletal α-actin promoter has been shown to involve competitive interactions of SRF, and the multifunctional transcriptional activator YY1, with a CARG-containing muscle regulatory element contained within the skeletal α-actin promoter (99, 156).

In summary, the preceding results indicate that *1*) transcriptional expression of smooth muscle α-actin requires the interaction of the CARG boxes with SMC nucleoprotein(s); *2*) skeletal myotubes, which express the smooth muscle α-actin gene, require a different repertoire of DNA elements and presumably nuclear factors than do SMCs to regulate the gene; and *3*) negative-acting *cis*-elements act to restrict transcription in non-SMCs that do not normally express smooth muscle α-actin. Results also clearly establish the utility of the smooth muscle α-actin promoter as a powerful tool with which to identify transcription factors important in control of SMC differentiation.

Results of the preceding studies have also established that constructs containing 547 bp (or more) of the region 5′ to the smooth muscle α-actin transcription start site conferred tissue-specific expression in cultured cells. Ongoing studies are in progress to determine whether these promoter constructs also confer cell type-specific expression in vivo. If so, these DNA regulatory sequences may have utility for constructing vectors for targeting gene therapies to the vasculature and for doing SMC-specific gene knockouts in mice.

Unfortunately, the promoters of additional SMC differentiation genes have not been cloned and characterized. As such, it is not possible at the present time to address the key question as to how SMCs coordinately control the expression of the family of genes that are necessary for its differentiated function. It is also important to note that not all genes characteristic of differentiated SMCs appear to be coordinately regulated during development (see sects. III–V), at least at the protein level. This, and the fact that differentiation in SMCs appears to be more highly dependent on environmental cues than in skeletal muscle, implies that if master differentiation control genes exist in SMCs, they presumably are more restrictive in the number of target genes that they affect as compared with the MyoD family. Note that the alternative possibility is that transcription of the entire family of genes necessary for differentiated function in SMCs is regulated independently. Whereas this possibility cannot be ruled out, it seems highly unlikely given the extremely complex nature of contractile function in SMCs and its dependence on a large number of proteins being expressed at the right time and at the appropriate levels. As such, it seems highly likely that differentiation control in SMCs will involve at least some "master differentiation control genes." However, as yet, none has been identified, and much more work is needed in this important area.

B. Posttranscriptional Control of Smooth Muscle Cell Differentiation

There is also considerable evidence that regulation of smooth muscle-specific gene expression involves posttranscriptional controls. As noted in section III, the smooth muscle isoforms of MHC as well as the nonmuscle

and smooth muscle variants of caldesmon, MLCs, vinculin, metavinculin, and tropomyosin are generated by alternative splicing of a single gene. As such, an understanding of the mechanisms that control this splicing are likely to be extremely important in understanding molecular regulation of SMC differentiation/maturation. However, these control processes are not well understood, and to my knowledge, there are few groups working in this very important area.

There is also evidence that regulation of mRNA turnover may play an important role in control of SMC differentiation. For example, work in our laboratory has shown that the marked decrease in smooth muscle α-actin expression following acute treatment of cultured rat aortic SMCs with PDGF-BB was not due to diminished transcription but rather to transcriptional-dependent destabilization of smooth muscle α-actin mRNA (50, 51). Little is known concerning sequence elements that may contribute to posttranscriptional regulation of actin mRNAs. However, there are a number of examples in which mRNA abundance is regulated exclusively by control of mRNA stability rather than by alterations in transcriptional rates (101, 207), and several structural determinants that regulate mRNA stability have been identified (128, 244). Regulation of transferrin receptor mRNA abundance is tightly coupled to the availability of iron. This regulation is dependent on the presence of a stem-loop structure in the 3′-untranslated region of the mRNA (294). In the presence of iron, the stem-loop structure forms and the mRNA is destabilized, resulting in a decrease in transferrin receptors in the presence of high concentrations of iron. We identified several energetically favorable stem-loop structures in the 3′-untranslated region of the smooth muscle α-actin mRNA (50). This raises the possibility that formation or disruption of this stem loop may be important to PDGF-induced accelerated smooth muscle α-actin mRNA decay. However, identification of the mRNA elements which confer PDGF-induced destabilization of smooth muscle α-actin mRNA will require further studies using site-directed mutagenesis.

C. *Additional Strategies for Identifying Smooth Muscle Cell Differentiation Control Genes*

An alternative strategy (other than direct studies of gene expression) that has been employed in an attempt to isolate differentiation control genes for vascular SMCs has been to look for genes that are homologous to known differentiation control genes found in other cell types. Despite extensive efforts in our laboratory and others, no MyoD or MyoD-related family members have been found in SMCs on the basis of screening of Northern blots as well as SMC cDNA libraries. The only exception is that SMCs, like many cell types, have been found to express the MyoD inhibitor Id (136). The function of Id in SMCs is unknown. However, there is no evidence at present implicating a role for it in control of SMC differentiation.

A number of genes are expressed in vascular SMCs that contain regions that are highly homologous to homeobox genes that were first identified in genetic studies in *Drosophila* and shown to be important in control of development of body form (for a review, see Ref. 162). Homeobox genes code for proteins that bind to specific nucleotide sequences in DNA and either activate or inhibit the corresponding genes (147). Homeobox proteins are related to one another primarily in the sequence of the 60-amino acid residue DNA binding site portion of the protein, the homeodomain.

Three homeodomain-containing genes have been shown to be expressed in vascular SMCs including *MHox* (20, 52). *Hox 1.11* (201), and *Gax* (94). *MHox* was initially cloned by Olson and co-workers (52) on the basis of its binding to an A+T-rich region adjacent to a MEF-2 site within the skeletal muscle creatine phosphokinase enhancer core. It is expressed in a variety of mesodermally derived cells in the developing embryo including skeletal, cardiac, and smooth muscle, as well as by nonmuscle cells. *Hox 1.11* was cloned by Walsh and co-workers (201) from a SMC cDNA library using a degenerate oligonucleotide to the highly conserved 60-amino acid homeodomain region that is highly conserved within this gene family. During development, it is expressed in a variety of different tissues including in the neural tube, in the myelencephalon, in the brachial arches, and in the vessels leading to the heart. In the adult, however, it was found in the aorta and lung, but not in cardiac muscle, skeletal muscle, intestinal smooth muscle, or brain. This same group (201) also identified a second homeobox gene designated *Gax*, whose expression is largely confined to the cardiovascular tissues of the adult (94). It shows rapid downregulation during G_0/G_1 transition in cultured vascular SMCs. Given the known role of many homeobox genes as transcription factors that are important in development (146), it is interesting to speculate that *MHox*, *Hox 1.11*, or *Gax* may play an important role in control of SMC differentiation. At present, however, the function of these genes in smooth muscle is not known, and there is no direct evidence that they are involved in control of either SMC differentiation or in transcriptional regulation in SMCs.

An additional approach that has been employed to identify SMC differentiation control genes has been to develop inducible SMC lineage systems analogous to the 10T1/2 cell system used by Weintraub and co-workers (57, 267) to identify MyoD. Using differential cDNA screening techniques, these workers identified genes (cDNAs) that were present in differentiated myotubes derived from 10T1/2 cells treated with the DNA-hypomethylating agent 5-azacytidine, but absent from the multipotential 10T1/2 fibroblasts. We have recently described a system in which multipotential P19 embryonal carcinoma cells are induced to form a smooth muscle lineage by treatment with retinoic acid (20). Clonal lines derived from retinoic acid-treated P19 cells were shown to express smooth muscle α-actin and smooth muscle MHC, as well as responsiveness to a number of contractile agonists including angiotensin II, norepinephrine, and endothelin. In contrast, multipotential parental P19 cells lacked these prop-

erties. This system should have utility in attempting to identify SMC differentiation/lineage control genes. However, it is important to emphasize that the value of the P19 system is unlikely to be in the retinoic acid-derived SMC lines themselves, which are likely to have many of the same properties as primary SMC culture lines with respect to expression of differentiated properties. Rather, the key value of the system is the demonstration that multipotential P19 cells can be stimulated to undergo determination/differentiation to a SMC lineage upon retinoic acid treatment. This implies *1*) that a cDNA encoding a regulatory protein for SMC differentiation/determination might be isolated by subtractive cloning techniques using this cell system and *2*) that any proteins identified (by this system or any other means) could be forcibly overexpressed in P19 cells to test their ability in inducing SMC determination/differentiation, much as overexpression of MyoD in 10T1/2 cells was shown to convert them to the skeletal muscle lineage (57). A limitation of this cell system is that unlike the azacytidine-10T1/2 system used by Davis et al. (57), cells at an intermediate, committed, but undifferentiated state, were not identified. Rather, the retinoic acid-derived SMC lines showed spontaneous differentiation, at least with respect to expression of smooth muscle α-actin and smooth muscle MHC and contractile agonist responsiveness.

IX. ALTERATIONS IN THE SMOOTH MUSCLE CELL'S DIFFERENTIATED STATE IN VASCULAR DISEASE

There is unequivocal evidence demonstrating that SMCs within human atherosclerotic lesions and myointimal lesions of experimental animals following vascular injury show an altered differentiated phenotype as compared with normal medial SMCs (89, 139, 141, 170). This process is characterized by extensive alterations in the morphological appearance of the cells, including a reduction in myofilaments, and an increase in synthetic cellular organelles such as Golgi and rough endoplasmic reticulum. In addition, intimal SMCs show reduced levels of a variety of proteins characteristic of differentiated SMCs including smooth muscle α-actin, smooth muscle MHC, caldesmon, vinculin, and desmin (85, 89, 139, 141, 170, 293). On the basis of studies in vascular injury models in animals, the changes in myointimal SMCs are at least partially reversible in that expression of smooth muscle contractile proteins increases in SMCs within chronic lesions (43, 139). Thus the changes may be a requisite part of the repair process, with early decreases reflecting a shift to a less differentiated state with an increased growth capacity (43), and later redifferentiation of the cells and return of contractile capabilities.

A key question is what controls the changes in the differentiated phenotype of the SMCs within intimal lesions? Results of studies in cultured SMCs showing that growth and differentiation are not necessarily mutually exclusive suggest that phenotypic modulation of intimal SMCs cannot be attributed solely to increased growth. Consistent with this, there is a very poor correlation between proliferation rates in intimal SMCs and their modified differentiated phenotype in that studies in both humans and experimental animal models have shown that alterations in the differentiated properties of intimal SMCs persist even in cases where proliferation rates have returned to normal values (43, 92, 187). A likely possibility is that the altered phenotype of lesion SMCs is at least in part a consequence of changes in the cellular environment that occur within the intima versus the media, including well-demonstrated changes in the extracellular matrix, growth factor expression, and lipid composition (see Refs. 30, 93 for reviews).

A related question is, Are the changes in the SMC differentiated state within intimal lesions the cause or the consequence of the disease process? Although additional studies are needed, it seems likely that both are correct; that is, the SMC is likely to change its phenotype in response to altered environmental cues that initiate the disease process. This could include many of the known atherogenic risk factors such as lipid peroxidation products, cellular toxins resulting from cigarette smoke, and endothelial injury or dysfunction that are as yet poorly understood (for reviews, see Refs. 30, 93, 213, 214). The altered SMC in turn exhibits characteristics that are likely to exacerbate lesion development. For example, there is an abundance of evidence showing altered lipid metabolism, growth factor production, and extracellular matrix production in intimal versus medial SMCs both in vivo as well as in cultured SMCs derived from these two sources (see reviews in Refs. 30, 213, 214). As such, the failure to appropriately regulate the differentiated phenotype of the vascular SMCs is likely a major contributing factor to lesion development and progression. A key question is, What are the critical signals that are necessary for maintenance of the differentiated phenotype of the SMC? As is evident at this late point in this review, these are not well defined, and much remains to be done in this important area.

An additional critical question is, What is the origin of the SMC that gives rise to the intimal lesion? Does it involve modification of medial SMCs that were fully differentiated, or is there a subpopulation of relatively undifferentiated SMCs that preexists in the normal blood vessel? It should be noted that the demonstration of the monoclonality of many human atherosclerotic lesions in studies by Benditt and Benditt (13) is not incompatible with either of these possibilities, since their data addressed whether lesion SMCs were derived from single versus multiple foci and in no way addressed the differentiated properties of the SMCs of origin. While the issue of whether relatively undifferentiated SMCs may exist within the normal media and give rise to lesion cells is not totally resolved, there is an abundance of morphological and immunocytological evidence that indicates that there is not a distinct subpopulation of SMCs in the media of normal adult human arteries that could be categorized as an undifferentiated SMC stem cell (1, 73). Moreover, studies by Clowes and Schwartz (45) in a rat carotid artery injury model have demonstrated that up to 40–50% of medial SMCs proliferate following injury

of the rat carotid artery. These results indicate that a large fraction of medial SMCs is capable of contributing to intimal lesion formation, which is clearly incompatible with the hypothesis that intimal SMCs must be derived from a rare and relatively undifferentiated SMC stem cell population, at least in this animal model. As noted previously in section V, there is clear evidence for heterogeneity in expression of SMC differentiation markers during vascular development (9, 73, 84, 197), as well as in atherosclerotic animal models (226, 293). For example, Zanellato et al. (293) found evidence for altered expression of MHC isoforms in medial SMCs of cholesterol-fed rabbits. Whereas medial SMCs in control rabbits stained exclusively with antibodies specific for SM-1 MHC and SM-2 MHC, a subpopulation of medial SMCs in the innermost layers of the aorta was observed in cholesterol-fed rabbits that showed loss of staining with a SM-2 MHC antibody but stained with a nonmuscle MHC antibody. Similarly, heterogeneity in vimentin and desmin expression within intimal SMCs has been reported following balloon injury of the rat aorta (76).

To summarize, most evidence suggests that intimal SMCs are derived from fully differentiated SMCs that undergo changes in their differentiated state in response to injury or atherogenic stimuli, rather than selective recruitment from a preexisting undifferentiated SMCs stem cell population. Changes are potentially reversible and are reflective of the extreme plasticity of the SMCs differentiated state.

In contrast to the profound changes in the differentiated phenotype of SMCs that occur in association with human or experimental atherosclerosis and vessel injury, hypertensive vascular disease, at least in the early stages of mild to moderate hypertension, is not associated with major alterations in the differentiated state of the vascular SMCs (see Ref. 191 for a review). Extensive ultrastructural studies have shown that the morphological appearance of medial arterial SMCs is not significantly different between hypertensives and controls. Consistent with these observations, we (194) and others (see Ref. 60 for a review) have shown that whereas there are quantitative increases in contractile proteins in hypertensive SMCs, there are not major qualitative changes, i.e., there is a proportionate increase in the contractile proteins relative to total cell protein. There are reports in the literature indicating that changes in SMC contractility occur in a variety of hypertensive models, although some of the purported changes may not represent a true change in contractility, but rather may have been due to alterations in vessel mass, geometry, and/or length-tension relationships (110, 171). In any event, they represent very subtle changes in the differentiated state of the SMCs as compared with changes that occur in atherogenesis.

It has been suggested that the increased growth in hypertensive vessels may represent an adaptive mechanism to normalize increases in wall stress as a result of elevations in blood pressure (172, 191); that is, from a teleological standpoint, one could argue that it would be advantageous for the organism to increase the contractile mass of the vessel commensurate with increased load, but without the at least temporary loss of contractile proteins and function characteristic of SMCs undergoing reparative growth. However, there is no conclusive evidence in this regard, and the failure to see significant alterations in contractile protein expression in hypertensive models may reflect the greatly diminished rate of SMC growth in hypertension as compared with that typically associated with injury models (194).

These differences in the pattern of changes in contractile protein expression in hypertension as compared with atherogenesis may also be attributed to the nature of the SMC growth responses. Accelerated growth of SMCs in human or experimental models of atherosclerosis is due primarily to intimal migration and proliferation of SMCs, whereas medial hypertrophy in large vessels of animals with chronic hypertension is due almost exclusively to enlargement of preexisting SMCs or cellular hypertrophy (reviewed in Ref. 191). Alternatively, differences may reflect influences of factors associated with the major site of the increased growth, i.e., the intima versus the media. Acute onset models of hypertension in which blood pressure rises to very high levels within several days are associated with evidence of endothelial cell injury that is not seen in slowly developing chronic models of hypertension (195, 202). In these models, there is significant intimal migration and proliferation of SMCs and evidence of phenotypic modulation similar to that which occurs with endothelial denudation models of vascular injury (202). Thus the nature of the changes in SMC differentiation that occur with hypertension, as well as the nature of the SMC growth response, varies depending on the particular model of hypertension studied.

X. SUMMARY AND FUTURE DIRECTIONS

In this review I have attempted to summarize our current knowledge of the regulation of differentiation of the vascular SMC. Studies have established that vascular SMCs express a unique repertoire of contractile proteins, ion channels, and signaling molecules that are required for their principle differentiated function in mature animals, which is contraction. However, the mature differentiated SMC also retains remarkable plasticity, such that it can undergo relatively rapid and reversible changes in its phenotype in response to a variety of different stimuli. Consistent with this property, differentiation/maturation of the vascular SMCs appears to be more highly dependent on environmental cues than in cells such as skeletal muscle which undergo terminal differentiation and which exhibit a much more restrictive differentiated phenotype. Much remains to be learned, particularly with regard to understanding the molecular mechanisms that control the differentiation program in vascular SMCs, as well as to identify the key environmental cues that are critical for inducing SMC differentiation/maturation. Additional studies are needed in many areas including the following: *1*) identification of additional SMC differentiation markers, particularly those whose developmental regulation is at the tran-

scriptional level; *2*) further investigation of the temporal patterns of expression of SMC differentiation markers that occur during vasculogenesis; and *3*) identification of the molecular mechanisms that control expression of SMC differentiation genes and in particular mechanisms responsible for controlling the coordinate regulation of these genes. Considerable progress has been made in identifying many of the changes that occur in the differentiated state of the SMCs with vascular injury and vascular disease. Indeed, in so much that many cultured SMC studies may have been done in cells that retained few differentiated functions of the SMCs, in many respects we may know more about the pathological phenotype of the SMCs than we do about its normal state. Progress has also been made in identifying SMC culture systems in which cells continue to express many of the known SMC differentiation markers. These SMC cultures will provide very valuable systems with which to identify potential regulatory pathways and molecules involved in control of SMC differentiation. However, cultured SMCs are clearly modified as compared with their in vivo counterparts, and as such, use of transgenic and gene knockout strategies are likely to play a key role in efforts to understand SMCs differentiation/maturation in vivo. An understanding of the cellular and molecular mechanisms that control SMC differentiation will be critical to understanding not only normal vascular development, but also a diverse range of major diseases in humans that are characterized by alterations in growth and differentiation of the SMCs, including atherosclerosis, hypertension, and cancer that account for over 75% of all deaths.

NOTE ADDED IN PROOF

Katoh et al. (Katoh, Y., E. Loukianov, E. Kopras, A. Zilberman, and M. Periasamy. Identification of functional promotor elements in the rabbit smooth muscle myosin heavy chain gene. *J. Biol. Chem.* 269: 30538–30545, 1994) recently reported the sequence and partial characterization of the rabbit SM MHC promoter.

This work was supported by National Heart, Lung, and Blood Institute Grants R01-HL-38854 and P01-HL-19242.

REFERENCES

1. AIKAWA, M., P. N. SIVAM, M. KURO-O, K. KIMURA, K. NAKAHARA, S. TAKEWAKI, M. UEDA, H. YAMAGUCHI, Y. YAZAKI, M. PERIASAMY, AND R. NAGAI. Human smooth myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis. *Circ. Res.* 73: 1000–1012, 1993.
2. APPLEGATE, D., W. FENG, R. S. GREEN, AND M. B. TAUBMAN. Cloning and expression of a novel acidic calponin isoform from rat aortic vascular smooth muscle. *J. Biol. Chem.* 269: 10683–10690, 1994.
3. ARCINIEGAS, E., A. B. SUTTON, T. D. ALLEN, AND A. M. SCHOR. Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. *J. Cell Sci.* 103: 521–529, 1992.
4. ASSOIAN, R. K., AND M. B. SPORN. Type beta transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. *J. Cell Biol.* 102: 1217–1223, 1986.
5. BABIJ, P. Tissue-specific and developmentally regulated alternative splicing of a visceral isoform of smooth muscle myosin heavy chain. *Nucleic Acids Res.* 21: 1467–1471, 1993.
6. BABIJ, P., S. KAWAMOTO, S. WHITE, R. S. ADELSTEIN, AND M. PERIASAMY. Differential expression of SM-1 and SM-2 myosin isoforms in cultured vascular smooth muscle. *Am. J. Physiol.* 262 (*Cell Physiol.* 31): C607–C613, 1992.
7. BADER, B. L., L. JAHN, AND W. W. FRANKE. Low level expression of cytokeratins 8, 18 and 19 in vascular smooth muscle cells of human umbilical cord and in cultured cells derived therefrom, with an analysis of the chromosomal locus containing the cytokeratin 19 gene. *Eur. J. Cell Biol.* 47: 300–319, 1988.
8. BARINAGA, M. Knockout mice: round two. *Science Wash. DC* 265: 26–28, 1994.
9. BARJA, F., C. COUGHLIN, D. BELIN, AND G. GABBIANI. Actin isoform synthesis and mRNA levels in quiescent and proliferating rat aortic smooth muscle cells in vivo and in vitro. *Lab. Invest.* 55: 226–233, 1986.
10. BARRETT, T. B., C. M. GAJDUSEK, S. M. SCHWARTZ, J. K. MCDOUGALL, AND E. P. BENDITT. Expression of the *sis* gene by endothelial cells in culture and in vivo. *Proc. Natl. Acad. Sci. USA* 81: 6772–6774, 1984.
11. BEAN, B. P., AND D. D. FRIEL. ATP-activated channels in excitable cells. *Ion Channel* 2: 169–203, 1990.
12. BELKIN, A. M., O. I. ORNATSKY, A. E. KABAKOV, M. A. GLUKHOVA, AND V. E. KOTELIANSKY. Diversity of vinculin/meta-vinculin in human tissues and cultivated cells. Expression of muscle specific variants of vinculin in human aorta smooth muscle cells. *J. Biol. Chem.* 263: 6631–6635, 1988.
13. BENDITT, E. P., AND J. M. BENDITT. Evidence for the monoclonal origin of human atherosclerotic plaques. *Proc. Natl. Acad. Sci. USA* 70: 1753–1756, 1973.
14. BENNETT, G. S., S. A. FELLINI, J. M. CROOP, J. J. OTTO, J. BRYAN, AND H. HOLTZER. Differences among 100-angstrom filament subunits from different cell types. *Proc. Natl. Acad. Sci. USA* 75: 4364–4368, 1978.
15. BERK, B. C., V. VEKSHTEIN, H. GORDON, AND T. TSUDA. Angiotensin II stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension Dallas* 13: 305–314, 1989.
16. BEVAN, R. D., AND H. TSURU. Functional and structural changes in the rabbit ear artery after sympathetic denervation. *Circ. Res.* 49: 478–485, 1981.
17. BIRUKOV, K. G., O. V. STEPANOVA, A. K. NANAEV, AND V. P. SHIRINSKY. Expression of calponin in rabbit and human aortic smooth muscle cells. *Cell Tissue Res.* 266: 579–584, 1991.
18. BLANK, R. S., T. C. MCQUINN, K. C. YIN, M. M. THOMPSON, K. TAKEYASU, R. J. SCHWARTZ, AND G. K. OWENS. Elements of the smooth muscle alpha-actin promoter required in *cis* for transcriptional activation in smooth muscle. Evidence for cell type-specific regulation. *J. Biol. Chem.* 267: 984–989, 1992.
19. BLANK, R. S., AND G. K. OWENS. Platelet-derived growth factor regulates actin isoform expression and growth state in cultured rat aortic smooth muscle cells. *J. Cell. Physiol.* 142: 635–642, 1990.
20. BLANK, R. S., E. A. SWARTZ, M. M. THOMPSON, E. N. OLSON, AND G. K. OWENS. A retinoic acid-induced clonal cell line derived from P19 embryonal carcinoma cells expresses smooth muscle characteristics. *Circ. Res.* 76: 742–749, 1995.
21. BLANK, R. S., M. M. THOMPSON, AND G. K. OWENS. Cell cycle versus density dependence of smooth muscle alpha actin expression in cultured rat aortic smooth muscle cells. *J. Cell Biol.* 107: 299–306, 1988.
22. BLAU, H., AND D. BALTIMORE. Differentiation requires continuous regulation. *J. Cell Biol.* 112: 781–783, 1991.
23. BLAU, H., G. PAVLATH, E. HARDEMAN, C. CHIU, L. SIBERSTEIN, S. WEBSTER, S. MILLER, AND C. WEBSTER. Plasticity of the differentiated state. *Science Wash. DC* 230: 758–766, 1985.
24. BODIN, P., S. RICHARD, C. TRAVO, P. BERTA, J. C. STOCLET, S. PAPIN, AND P. TRAVO. Responses of subcultured rat aortic smooth

muscle myocytes to vasoactive agents and KCl-induced depolarization. *Am. J. Physiol.* 260 (*Cell Physiol.* 29): C151–C158, 1991.
25. BORRIONE, A. C., A. M. ZANELLATO, L. GIURIATO, G. SCANNAPIECO, P. PAULETTO, AND S. SARTORE. Nonmuscle and smooth muscle myosin isoforms in bovine endothelial cells. *Exp. Cell Res.* 190: 1–10, 1990.
26. BOXER, L. M., R. PRYWES, R. G. ROEDER, AND L. KEDES. The sarcomeric actin CArG-binding factor is indistinguishable from the c-*fos* serum response factor. *Mol. Cell. Biol.* 9: 515–522, 1989.
27. BRAUN, T., G. BUSCHHAUSEN-DENKER, E. BOBER, E. TANNICH, AND H. H. ARNOLD. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* 8: 701–709, 1989.
28. BRETSCHER, A. Thin filament regulatory proteins of smooth muscle and nonmuscle cells. *Nature Lond.* 321: 726–727, 1986.
29. BYRNE, B. J., Y. J. KACZOROWSKI, M. D. COUTU, AND S. W. CRAIG. Chicken vinculin and meta-vinculin are derived from a single gene by alternative splicing of a 207-base pair exon unique to meta-vinculin. *J. Biol. Chem.* 267: 12845–12850, 1992.
30. CAMPBELL, G. R., AND J. H. CAMPBELL. Smooth muscle phenotypic changes in arterial wall hemostasis: implications for the pathogenesis of atherosclerosis. *Exp. Mol. Pathol.* 42: 139–162, 1985.
31. CAMPBELL, J. H., AND G. R. CAMPBELL. Endothelial cell influences on vascular smooth muscle phenotype. *Annu. Rev. Physiol.* 48: 295–306, 1986.
32. CAMPBELL, J. H., O. KOCHER, O. SKALLI, G. GABBIANI, AND G. R. CAMPBELL. Cytodifferentiation and expression of alpha-smooth muscle actin mRNA and protein during primary culture of aortic smooth muscle cells. Correlation with cell density and proliferative state. *Arteriosclerosis* 9: 633–643, 1989.
33. CAREY, D. J. Control of growth and differentiation of vascular cells by extracellular matrix proteins. *Annu. Rev. Physiol.* 53: 161–177, 1991.
34. CARROLL, S. L., D. J. BERGSMA, AND R. J. SCHWARTZ. Structure and complete nucleotide sequence of the chicken alpha-smooth muscle (aortic) actin gene. An actin gene which produces multiple messenger RNAs. *J. Biol. Chem.* 261: 8965–8976, 1986.
35. CARROLL, S. L., D. J. BERGSMA, AND R. J. SCHWARTZ. A 29-nucleotide DNA segment containing an evolutionarily conserved motif is required in *cis* for cell-type-restricted repression of the chicken alpha-smooth muscle actin gene core promoter. *Mol. Cell. Biol.* 8: 241–250, 1988.
36. CASTELLOT, J. J., L. V. FAVREAU, M. J. KARNOVSKY, AND R. D. ROSENBERG. Inhibition of vascular smooth muscle cell growth by endothelial cell-derived heparin. Possible role of a platelet endoglycosidase. *J. Biol. Chem.* 257: 11256–11260, 1982.
37. CAULFIELD, M. P. Muscarinic receptors: characterization, coupling and function. *Pharmacol. Ther.* 58: 319–379, 1993.
38. CHAMLEY, J. H., G. R. CAMPBELL, AND G. BURNSTOCK. Dedifferentiation, redifferentiation and bundle formation of smooth muscle cells in tissue culture: the influence of cell number and nerve fibres. *J. Embryol. Exp. Morphol.* 32: 297–323, 1974.
39. CHAMLEY-CAMPBELL, J. H., AND G. R. CAMPBELL. What controls smooth muscle phenotype? *Atherosclerosis* 40: 347–357, 1981.
40. CHAMLEY-CAMPBELL, J. H., G. R. CAMPBELL, AND R. ROSS. The smooth muscle cell in culture. *Physiol. Rev.* 59: 1–6, 1979.
41. CINTORINO, M., E. BELLIZZI DE MARCO, P. LEONCINI, S. A. TRIPODI, L. J. XU, A. P. SAPPINO, A. SCHMITT GRAFF, AND G. GABBIANI. Expression of alpha-smooth-muscle actin in stromal cells of the uterine cervix during epithelial neoplastic changes. *Int. J. Cancer* 47: 843–846, 1991.
42. CLOWES, A. W., M. M. CLOWES, J. FINGERLE, AND M. A. REIDY. Regulation of smooth muscle cell growth in injured artery. *J. Cardiovasc. Pharmacol.* 14, *Suppl.* 6: S12–S15, 1989.
43. CLOWES, A. W., M. M. CLOWES, O. KOCHER, P. ROPRAZ, C. CHAPONNIER, AND G. GABBIANI. Arterial smooth muscle cells in vivo: relationship between actin isoform expression and mitogenesis and their modulation by heparin. *J. Cell Biol.* 107: 1939–1945, 1988.
44. CLOWES, A. W., M. M. CLOWES, AND M. A. REIDY. Kinetics of cellular proliferation after arterial injury. III. Endothelial and smooth muscle growth in chronically denuded vessels. *Lab. Invest.* 54: 295–303, 1986.
45. CLOWES, A. W., AND S. M. SCHWARTZ. Significance of quiescent smooth muscle migration in the injured rat carotid artery. *Circ. Res.* 56: 139–145, 1985.
46. CLYMAN, R. I., K. A. MCDONALD, AND R. H. KRAMER. Integrin receptors on aortic smooth muscle cells mediate adhesion to fibronectin, laminin, and collagen. *Circ. Res.* 67: 175–186, 1990.
47. COBB, J. L. S., AND T. BENNETT. An untrastructural study of mitotic division in differentiated gastric smooth muscle cells. *Z. Zellforsch.* 103: 177–189, 1970.
48. COFFIN, J. D., AND T. J. POOLE. Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* 102: 735–748, 1988.
49. COLLINS, T., J. S. POBER, M. A. GIMBRONE, JR., A. HAMMACHER, C. BETSHOLTZ, B. WESTERMARK, AND C. H. HELDIN. Cultured human endothelial cells express platelet-derived growth factor A chain. *Am. J. Pathol.* 126: 7–12, 1987.
50. CORJAY, M. H., R. S. BLANK, AND G. K. OWENS. Platelet-derived growth factor-induced destabilization of smooth muscle α-actin mRNA. *J. Cell. Physiol.* 145: 391–397, 1990.
51. CORJAY, M. H., M. M. THOMPSON, K. R. LYNCH, AND G. K. OWENS. Differential effect of platelet-derived growth factor- versus serum-induced growth on smooth muscle alpha-actin and nonmuscle beta-actin mRNA expression in cultured rat aortic smooth muscle cells. *J. Biol. Chem.* 264: 10501–10506, 1989.
52. CSERJESI, P., B. LILLY, L. BRYSON, Y. WANG, D. A. SASSOON, AND E. N. OLSON. MHox: a mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. *Development* 115: 1087–1101, 1992.
53. CSERJESI, P., AND E. N. OLSON. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. *Mol. Cell. Biol.* 11: 4854–4862, 1991.
54. DARBY, I., O. SKALLI, AND G. GABBIANI. Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab. Invest.* 63: 21–29, 1990.
55. DARNELL, J., H. LODISH, AND D. BALTIMORE. *Molecular Cell Biology.* New York: Freeman, 1986, p. 987–1035.
56. DARTSCH, P. C., H. HAMMERLE, AND E. BETZ. Orientation of cultured arterial smooth muscle cells growing on cyclically stretched substrates. *Acta Anat.* 125: 108–113, 1986.
57. DAVIS, R. L., H. WEINTRAUB, AND A. B. LASSAR. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51: 987–1000, 1987.
58. DE MEY, J. G., M. P. UITENDAAL, H. C. BOONEN, M. J. VRIJDAG, M. J. DAEMEN, AND H. A. STRUYKER-BOUDIER. Acute and long-term effects of tissue culture on contractile reactivity in renal arteries of the rat. *Circ. Res.* 65: 1125–1135, 1989.
59. DESMOULIERE, A., L. RUBBIA BRANDT, AND G. GABBIANI. Modulation of actin isoform expression in cultured arterial smooth muscle cells by heparin and culture conditions. *Arterioscler. Thromb.* 11: 244–253, 1991.
60. DOMINICZAK, A. F., AND D. F. BOHR. Vascular smooth muscle in hypertension. *J. Hypertens.* 7: S107–S115, 1989.
61. DRAKE, C. J., AND A. G. JACOBSON. A survey by scanning electron microscopy of the extracellular matrix and endothelial components of the primordial chick heart. *Anat. Rec.* 222: 391–400, 1988.
62. DREW, J. S., C. MOOS, AND R. A. MURPHY. Localization of isoactins in isolated smooth muscle thin filaments by double gold immunolabeling. *Am. J. Physiol.* 260 (*Cell Physiol.* 29): C1332–C1340, 1991.
63. DUBAND, J. L., A. M. BELKIN, J. SYFRIG, J. P. THIERY, AND V. E. KOTELIANSKY. Expression of alpha1 integrin, a laminin-collagen receptor, during myogenesis and neurogenesis in the avian embryo. *Development* 116: 585–600, 1992.
64. DUBAND, J. L., M. GIMONA, M. SCATENA, S. SARTORE, AND J. V. SMALL. Calponin and SM 22 as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. *Differentiation* 55: 1–11, 1993.
65. DUCKLES, S. P., AND W. BANNER. Changes in vascular smooth muscle reactivity during development. *Annu. Rev. Pharmcol. Toxicol.* 24: 65–83, 1984.

66. EBASHI, S., AND F. EBASHI. A new protein component participating in the superprecipitation of myosin B. *J. Biochem.* 55: 604–613. 1964.
67. EDMONDSON, D. G., AND E. N. OLSON. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev.* 3: 628–640, 1989.
68. ERDODI, F., M. BARANY, AND K. BARANY. Myosin light chain isoforms and their phosphorylation in arterial smooth muscle. *Circ. Res.* 61: 898–903, 1987.
69. FATIGATI, V., AND R. A. MURPHY. Actin and tropomyosin variants in smooth muscles. Dependence on tissue type. *J. Biol. Chem.* 259: 14383–14388, 1984.
70. FOLKMAN, J., AND Y. SHING. Control of angiogenesis by heparin and other sulfated polysaccharides. *Adv. Exp. Med. Biol.* 313: 355–364. 1992.
71. FOSTER, D. N., B. MIN, L. K. FOSTER, E. S. STOFLET, S. SUN, M. J. GETZ, AND A. R. STRAUCH. Positive and negative *cis*-acting regulatory elements mediate expression of the mouse vascular smooth muscle alpha-actin gene. *J. Biol. Chem.* 267: 11995–12003, 1992.
72. FRANKE, W. W., E. SCHMID, C. FREUDENSTEIN, B. APPELHANS, M. OSBORN, K. WEBER, AND T. W. KEENAN. Intermediate-sized filaments of the prekeratin type in myoepithelial cells. *J. Cell Biol.* 84: 633–654, 1980.
73. FRID, M. G., O. Y. PRINTESVA, A. CHIAVEGATO, E. FAGGIN, M. SCATENA, V. E. KOTELIANSKY, P. PAULETTO, M. A. GLUKHOVA, AND S. SARTORE. Myosin heavy-chain isoform composition and distribution in developing and adult human aortic smooth muscle. *J. Vasc. Res.* 30: 279–292, 1993.
74. FRID, M. G., B. V. SHEKHONIN, V. E. KOTELIANSKY, AND M. A. GLUKHOVA. Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Dev. Biol.* 153: 185–193, 1992.
75. FRITZE, L. M., C. F. REILLY, AND R. D. ROSENBERG. An antiproliferative heparan sulfate species produced by postconfluent smooth muscle cells. *J. Cell Biol.* 100: 1041–1049, 1985.
76. GABBIANI, G., E. RUNGGER-BRANDLE, C. DE CHASTONAY, AND W. W. FRANKE. Vimentin-containing smooth muscle cells in aortic intimal thickening after endothelial injury. *Lab. Invest.* 265: 265–269, 1982.
77. GABBIANI, G., E. SCHMID, S. WINTER, C. CHAPONNIER, C. DE CHASTONAY, J. VANDEKERCKHOVE, K. WEBER, AND W. FRANKE. Vascular smooth muscle cells differ from other smooth muscle cells: predominance of vimentin filaments and a specific-type actin. *Proc. Natl. Acad. Sci. USA* 78: 298–300, 1981.
78. GAJDUSEK, C. M., AND S. M. SCHWARTZ. Ability of endothelial cells to condition culture medium. *J. Cell. Physiol.* 110: 35–42, 1982.
79. GEIGER, B., K. T. TOKUYASU, A. H. DUTTON, AND S. J. SINGER. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. USA* 77: 4127–4131, 1980.
80. GIACHELLI, C. M., M. W. MAJESKY, AND S. M. SCHWARTZ. Developmentally regulated cytochrome *P*-450IA1 expression in cultured rat vascular smooth muscle cells. *J. Biol. Chem.* 266: 3981–3986, 1991.
81. GIMONA, M., M. HERZOG, J. VANDEKERCKHOVE, AND J. V. SMALL. Smooth muscle specific expression of calponin. *FEBS Lett.* 274: 159–162, 1990.
82. GIMONA, M., M. P. SPARROW, P. STRASSER, M. HERZOG, AND J. V. SMALL. Calponin and SM 22 isoforms in avian and mammalian smooth muscle: absence of phosphorylation in vivo. *Eur. J. Biochem.* 205: 1067–1075, 1992.
83. GIRARD, H. Arterial pressure in the chick embryo. *Am. J. Physiol.* 224: 454–460, 1973.
84. GIURIATO, L., M. SCATENA, A. CHIAVEGATO, M. TONELLO, G. SCANNAPIECO, P. PAULETTO, AND S. SARTORE. Non-muscle myosin isoforms and cell heterogeneity in developing rabbit vascular smooth muscle. *J. Cell Sci.* 101: 233–246, 1992.
85. GIURIATO, L., M. SCATENA, A. CHIAVEGATO, A. M. ZANELLATO, D. GUIDOLIN, P. PAULETTO, AND S. SARTORE. Localization and smooth muscle cell composition of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits. *Arterioscler. Thromb.* 13: 347–359, 1993.
86. GLUKHOVA, M., V. KOTELIANSKY, C. FONDACCI, F. MAROTTE, AND L. RAPPAPORT. Laminin variants and integrin laminin receptors in developing and adult human smooth muscle. *Dev. Biol.* 157: 437–447. 1993.
87. GLUKHOVA, M. A., M. G. FRID, AND V. E. KOTELIANSKY. Developmental changes in expression of contractile and cytoskeletal proteins in human aortic smooth muscle. *J. Biol. Chem.* 265: 13042–13046. 1990.
88. GLUKHOVA, M. A., A. E. KABAKOV, A. M. BELKIN, M. G. FRID, O. I. ORNATSKY, N. I. ZHIDKOVA, AND V. E. KOTELIANSKY. Metavinculin distribution in adult human tissues and cultured cells. *FEBS Lett.* 207: 139–141, 1986.
89. GLUKHOVA, M. A., A. E. KABAKOV, M. G. FRID, O. I. ORNATSKY, A. M. BELKIN, D. N. MUKHIN, A. N. OREKHOV, V. E. KOTELIANSKY, AND V. N. SMIRNOV. Modulation of human aorta smooth muscle cell phenotype: a study of muscle-specific variants of vinculin, caldesmon, and actin expression. *Proc. Natl. Acad. Sci. USA* 85: 9542–9546, 1988.
90. GOMEZ, R. A., K. R. LYNCH, B. C. STURGILL, J. P. ELWOOD, R. L. CHEVALIER, R. M. CAREY, AND M. J. PEACH. Distribution of renin mRNA and its protein in the developing kidney. *Am. J. Physiol.* 257 (*Renal Fluid Electrolyte Physiol.* 26): F850–F858, 1989.
91. GONZALEZ-CRUSSI, F. Vasculogenesis in the chick embryo. An ultrastructural study. *Am. J. Anat.* 130: 441–460, 1971.
92. GORDON, D., M. A. REIDY, E. P. BENDITT, AND S. M. SCHWARTZ. Cell proliferation in human coronary arteries. *Proc. Natl. Acad. Sci. USA* 87: 4600–4604, 1990.
93. GORDON, D., AND S. M. SCHWARTZ. Replication of arterial smooth muscle cells in hypertension and atherosclerosis. *Am. J. Cardiol.* 59: 44–48, 1987.
94. GORSKI, D. H., D. F. LEPAGE, C. V. PATEL, N. G. COPELAND, N. A. JENKINS, AND K. WALSH. Molecular cloning of a diverged homeobox gene that is rapidly downregulated during the G_0/G_1 transition in vascular smooth muscle cells. *Mol. Cell. Biol.* 13: 3722–3733, 1993.
95. GRANT, D. S., K. TASHIRO, B. SEGUI REAL, Y. YAMADA, G. R. MARTIN, AND H. K. KLEINMAN. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. *Cell* 58: 933–943, 1989.
96. GRONE, H. J., M. SIMON, AND E. FUCHS. Autoradiographic characterization of angiotensin receptor subtypes in fetal and adult human kidney. *Am. J. Physiol.* 262 (*Renal Fluid Electrolyte Physiol.* 31): F326–F331, 1992.
97. GRUNWALD, J., A. V. CHOBANIAN, AND C. C. HAUDENSCHILD. Smooth muscle cell migration and proliferation: atherogenic mechanisms in hypertension. *Atherosclerosis* 67: 215–221, 1987.
98. GU, H., J. D. MARTH, P. C. ORBAN, H. MOSSMAN, AND K. RAJEWSKY. Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science Wash. DC* 265: 103–106, 1994.
99. GUALBERTO, A., D. LEPAGE, G. PONS, S. L. MADER, K. PARK, M. L. ATCHISON, AND K. WALSH. Functional antagonism between YY1 and the serum response factor. *Mol. Cell. Biol.* 12: 4209–4216. 1992.
100. GUNNING, P., M. GORDON, R. WADE, R. GAHLMANN, C.-S. LIN, AND E. HARDEMAN. Differential control of tropomyosin mRNA levels during myogenesis suggests the existence of an isoform competition-autoregulatory compensation control mechanism. *Dev. Biol.* 138: 443–453, 1990.
101. HAMALAINEN, L., J. OIKARINEN, AND K. I. KIVIRIKKO. Synthesis and degradation of type I procollagen mRNAs in cultured human skin fibroblasts and effect of cortisol. *J. Biol. Chem.* 260: 720–725. 1985.
102. HAMBURGER, V., AND H. L. HAMILTON. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88: 49–92. 1951.
103. HAN, D. K., AND G. LIAU. Identification and characterization of developmentally regulated genes in vascular smooth muscle cells. *Circ. Res.* 71: 711–719, 1992.

104. HANNAN, R. L., S. KOUREMBANAS, K. C. FLANDERS, S. J. ROGELJ, A. B. ROBERTS, D. V. FALLER, AND M. KLAGSBRUN. Endothelial cells synthesize basic fibroblast growth factor and transforming growth factor beta. *Growth Factors* 1: 7–17, 1988.
105. HART, P. J., K. E. OVERTURF, S. N. RUSSELL, A. CARL, J. R. HUME, K. M. SANDERS, AND B. HOROWITZ. Cloning and expression of a Kv1.2 class delayed rectifier K^+ channel from canine colonic smooth muscle. *Proc. Natl. Acad. Sci. USA* 90: 9659–9663, 1993.
106. HASEGAWA, Y., Y. UEDA, M. WATANABE, AND F. MORITA. Studies on amino acid sequences of two isoforms of 17-kDa essential light chain of smooth muscle myosin from porcine aorta media. *J. Biochem.* 111: 798–803, 1992.
107. HASTY, P., A. BRADLEY, J. H. MORRIS, D. G. EDMONDSON, J. M. VENUTI, E. N. OLSON, AND W. H. KLEIN. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature Lond.* 364: 501–506, 1993.
108. HAUDENSCHILD, C. C., AND J. GRUNWALD. Proliferative heterogeneity of vascular smooth muscle cells and its alteration by injury. *Exp. Cell Res.* 157: 364–370, 1985.
109. HAYASHI, K., Y. FUJIO, I. KATO, AND K. SOBUE. Structural and functional relationships between H- and L-caldesmons. *J. Biol. Chem.* 266: 355–361, 1991.
110. HEAGERTY, A. M., C. AALKJAER, A. J. BUND, N. KORSGAARD, AND M. J. MULVANY. Small artery structure in hypertension: dual processes of remoldeling and growth. *Hypertension Dallas* 21: 391–397, 1993.
111. HEDIN, U., B. A. BOTTGER, J. LUTHMAN, S. JOHANSSON, AND J. THYBERG. A substrate of the cell-attachment sequence of fibronectin (Arg-Gly-Asp-Ser) is sufficient to promote transition of arterial smooth muscle cells from a contractile to a synthetic phenotype. *Dev. Biol.* 133: 489–501, 1989.
112. HELPER, D. J., J. A. LASH, AND D. R. HATHAWAY. Distribution of isoelectric variants of the 17,000-dalton myosin light chain in mammalian smooth muscle. *J. Biol. Chem.* 263: 15748–15753, 1988.
113. HERMSMEYER, K. Calcium channel function in hypertension. *J. Hum. Hypertens.* 7: 173–176, 1993.
114. HILL, C. S., R. MARAIS, S. JOHN, J. WYNNE, S. DALTON, AND R. TREISMAN. Functional analysis of a growth factor-responsive transcription factor complex. *Cell* 73: 395–406, 1993.
115. HIRAKOW, R., AND T. HIRUMA. Scanning electron microscopic study on the development of primitive blood vessels in chick embryos at the early somite stage. *Anat. Embryol.* 163: 299–306, 1981.
116. HOLYCROSS, B. J., R. S. BLANK, M. M. THOMPSON, M. J. PEACH, AND G. K. OWENS. Platelet-derived growth factor-BB-induced suppression of smooth muscle cell differentiation. *Circ. Res.* 71: 1525–1532, 1992.
117. HOLYCROSS, B. J., M. J. PEACH, AND G. K. OWENS. Angiotensin II stimulates increased protein synthesis, not increased DNA synthesis, in intact rat aortic segments, in vitro. *J. Vasc. Res.* 30: 80–86, 1993.
118. HOOD, L. C., AND T. H. ROSENQUIST. Coronary artery development in the chick: origin and deployment of smooth muscle cells, and the effects of neural crest ablation. *Anat. Rec.* 234: 291–300, 1992.
119. HOSOYA, M., J.-I. MIYAZAKI, AND T. HIRABAYASHI. Tropomyosin isoforms in developing chicken gizzard smooth muscle. *J. Biochem.* 105: 712–717, 1989.
120. HU, N., AND E. B. CLARK. Hemodynamics of the stage 12 to stage 29 chick embryo. *Circ. Res.* 65: 1665–1670, 1989.
121. HUMPHREY, M. B., H. HERRERA SOSA, G. GONZALEZ, R. LEE, AND J. BRYAN. Cloning of cDNAs encoding human caldesmons. *Gene* 112: 197–204, 1992.
122. HUNGERFORD, J. E., AND C. D. LITTLE. The developmental biology of vascular smooth muscle as studies with a new monoclonal antibody directed against an embryonic quail vascular smooth muscle antigen (Abstract). *J. Cell Biochem.* 17: 198, 1993.
123. HYNES, R. O. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell* 69: 11–25, 1992.
124. IMAI, H., K. J. LEE, S. K. LEE, K. T. LEE, R. M. ONEAL, AND W. A. THOMAS. Ultrastructural features of aortic cell in mitosis in control and cholesterol-fed swine. *Lab. Invest.* 23: 401–415, 1979.
125. ISENBERG, G. Nonselective cation channels in cardiac and smooth muscle cells. *EXS* 66: 247–260, 1993.
126. JANSON, L. W., J. R. SELLERS, AND D. L. TAYLOR. Actin-binding proteins regulate the work performed by myosin II motors on single actin filaments. *Cell Motil. Cytoskeleton* 22: 274–280, 1995.
127. JOSEPH SILVERSTEIN, J., AND D. B. RIFKIN. Endothelial cell growth factors and the vessel wall. *Semin. Thromb. Hemostasis* 13: 504–513, 1987.
128. KABNICK, K. S., AND D. E. HOUSMAN. Determinants that contribute to cytoplasmic stability of human c-*fos* and β-globin mRNAs are located at several sites in each mRNA. *Mol. Cell. Biol.* 8: 3244–3520, 1988.
129. KAMM, K. E., AND J. T. STULL. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmcol. Toxicol.* 25: 593–620, 1985.
130. KANDA, K., T. MATSUDA, AND T. OKA. Mechanical stress induced cellular orientation and phenotypic modulation of 3-D cultured smooth muscle cells. *ASAIO J.* 39: M686–M690, 1994.
131. KATOH, N., AND S. KUBO. Light chains of chicken embryonic gizzard myosin. *Biochim. Biophys. Acta* 535: 401–411, 1978.
132. KAWAMOTO, S., AND R. S. ADELSTEIN. Characterization of myosin heavy chains in cultured smooth muscle cells. *J. Biol. Chem.* 262: 7282–7288, 1987.
133. KAWAMOTO, S., AND R. S. ADELSTEIN. Chicken nonmuscle myosin heavy chains: differential expression of two mRNAs and evidence for two different polypeptides. *J. Cell Biol.* 112: 915–924, 1991.
134. KAWASHIMA, M., Y. NABESHIMA, T. OBINATA, AND Y. FUJII-KURIYAMA. A common myosin light chain is expressed in chicken embryonic skeletal, cardiac, and smooth muscles and in brain continuously from embryo to adult. *J. Biol. Chem.* 262: 14408–14414, 1987.
135. KELLEY, C. A., M. TAKAHASHI, J. H. YU, AND R. S. ADELSTEIN. An insert of seven amino acids confers functional differences between smooth muscle myosins from the intestines and vasculature. *J. Biol. Chem.* 268: 12848–12854, 1993.
136. KEMP, P. R., D. J. GRAINGER, C. M. SHANAHAN, P. L. WEISSBERG, AND J. C. METCALFE. The Id gene is activated by serum but is not required for de-differentiation in rat vascular smooth muscle cells. *Biochem. J.* 277: 285–288, 1991.
137. KIM, J.-H., P. R. BUSHEL, AND C. C. KUMAR. Smooth muscle α-actin promoter activity is induced by serum stimulation of fibroblast cells. *Biochem. Biophys. Res. Commun.* 190: 1115–1121, 1993.
138. KIMES, B. W., AND B. L. BRANDT. Characterization of two putative smooth muscle cell lines from rat thoracic aorta. *Exp. Cell Res.* 98: 349–366, 1976.
139. KOCHER, O., F. GABBIANI, G. GABBIANI, M. A. REIDY, M. S. COKAY, H. PETERS, AND I. HUTTNER. Phenotypic features of smooth muscle cells during the evolution of experimental carotid artery intimal thickening. Biochemical and morphologic studies. *Lab. Invest.* 65: 459–470, 1991.
140. KOCHER, O., AND G. GABBIANI. Expression of actin mRNAs in rat aortic smooth muscle cells during development, experimental intimal thickening, and culture. *Differentiation* 32: 245–251, 1986.
141. KOCHER, O., AND G. GABBIANI. Cytoskeletal features of normal and atheromatous human arterial smooth muscle cells. *Hum. Pathol.* 17: 875–880, 1986.
142. KOTELIANSKY, V. E., E. P. OGRYZKO, N. I. ZHIDKOVA, P. A. WELLER, D. R. CRITCHLEY, K. VANCOMPERNOLLE, J. VANDEKERCKHOVE, P. STRASSER, M. WAY, M. GIMONA, AND J. V. SMALL. An additional exon in the human vinculin gene specifically encodes meta-vinculin-specific difference peptide. Cross-species comparison reveals variable and conserved motifs in the meta-vinculin insert. *Eur. J. Biochem.* 204: 767–772, 1992.
143. KURO-O, M., R. NAGAI, K. NAKAHARA, H. KATOH, R.-C. TSAI, H. TSUCHIMOCHI, Y. YAZAKI, A. OHKUBO, AND F. TAKAKU. cDNA cloning of a myosin heavy chain isoform in embryonic smooth muscle and its expression during vascular development and in arteriosclerosis. *J. Biol. Chem.* 266: 3768–3773, 1991.
144. KURO-O, M., R. NAGAI, H. TSUCHIMOCHI, H. KATOH, Y. YAZAKI, A. OHKUBO, AND F. TAKAKU. Developmentally regulated expres-

sion of vascular smooth muscle myosin heavy chain isoforms. *J. Biol. Chem.* 264: 18272–18275, 1989.
145. LANGILLE, B. L. Remodeling of developing and mature arteries: endothelium, smooth muscle, and matrix. *J. Cardiovasc. Pharmacol.* 21, *Suppl.* 1: S11–S17, 1993.
146. LATHAM, K. E., AND I. R. KONIGSBERG. Mitogen stimulation affects contractile protein mRNA abundance and translation in embryonic quail myocytes. *Mol. Cell. Biol.* 9: 3203–3211, 1989.
147. LAUGHON, A. DNA binding specificity of homeodomains. *Biochemistry* 30: 11357–11367, 1991.
148. LAZARD, D., X. SASTRE, M. G. FRID, M. A. GLUKHOVA, J. P. THIERY, AND V. E. KOTELIANSKY. Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. *Proc. Natl. Acad. Sci. USA* 90: 999–1003, 1994.
149. LEES-MILLER, J. P., AND D. M. HELFMAN. The molecular basis for tropomyosin isoform diversity. *Bioessays* 13: 429–437, 1991.
150. LELIEVRE, C. S., AND N. M. LEDOUARIN. Mesenchymal derivatives of the neural crest: analysis of chimeric quail and chick embryos. *J. Embryol. Exp. Morphol.* 34: 125–154, 1975.
151. LEMIRE, J. M., C. W. COVIN, S. WHITE, C. M. GIACHELLI, AND S. M. SCHWARTZ. Characterization of cloned aortic smooth muscle cells from young rats. *Am. J. Pathol.* 144: 1068–1081, 1994.
152. LENZ, S., P. LOHSE, U. SEIDEL, AND H. ARNOLD. The alkali light chains of human smooth muscle and nonmuscle myosins are encoded by a single gene. *J. Biol. Chem.* 264: 9009–9015, 1989.
153. LEUNG, D., S. GLAGOV, AND M. MATHEWS. A new in vitro method for studying cell responses to mechanical stimulation: different effects of cyclic stretching and agitation on smooth muscle cell biosynthesis. *Exp. Cell Res.* 109: 285–298, 1977.
154. LI, Z. L., A. LILIENBAUM, G. BUTLER-BROWNE, AND D. PAULIN. Human desmin-coding gene: complete nucleotide sequence, characterization and regulation of expression during myogenesis and development. *Gene* 78: 243–254, 1989.
155. LITTLE, C. D., D. M. PIQUET, L. A. DAVIS, L. WALTERS, AND C. J. DRAKE. Distribution of laminin, collagen type IV, collagen type I, and fibronectin in chicken cardiac jelly/basement membrane. *Anat. Rec.* 224: 417–425, 1989.
156. MACLELLAN, W. R., T. C. LEE, R. J. SCHWARTZ, AND M. D. SCHNEIDER. Transforming growth factor-beta response elements of the skeletal alpha-actin gene. Combinatorial action of serum response factor, YY1, and the SV40 enhancer-binding protein, TEF-1. *J. Biol. Chem.* 269: 16754–16760, 1994.
157. MALMQVIST, U., AND A. ARNER. Correlation between isoform composition of the 17 kDa myosin light chain and maximal shortening velocity in smooth muscle. *Pfluegers Arch.* 418: 523–530, 1990.
158. MANASEK, F. J. The ultrastructure of embryonic myocardial blood vessels. *Dev. Biol.* 26: 42–54, 1971.
159. MARAIS, R., J. WYNNE, AND R. TREISMAN. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* 73: 381–393, 1993.
160. MAROTTI, K. R., C. K. CASTLE, T. P. BOYLE, A. H. LIN, R. W. MURRAY, AND G. W. MELCHIOR. Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature Lond.* 364: 73–75, 1993.
161. MARSTON, S. B., AND C. J. W. SMITH. The thin filaments of smooth muscle. *J. Muscle Res. Cell Motil.* 6: 669–708, 1985.
162. McGINNIS, W., AND R. KRUMLAUF. Homeobox genes and axial patterning. *Cell* 68: 283–302, 1992.
163. McNAMARA, C. A., I. J. SAREMBOCK, L. W. GIMPLE, J. W. FENTON, S. R. COUGHLIN, AND G. K. OWENS. Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. *J. Clin. Invest.* 91: 94–98, 1993.
164. MIANO, J., P. CSERJESI, K. LIGON, M. PERISAMY, AND E. N. OLSON. Smooth muscle myosin heavy chain marks exclusively the smooth muscle lineage during mouse embryogenesis. *Circ. Res.* 75: 803–812, 1994.
165. MIN, B., D. N. FOSTER, AND A. R. STRAUCH. The 5′-flanking region in the mouse vascular smooth muscle α-actin gene contains evolutionary conserved motifs within a functional promoter. *J. Biol. Chem.* 265: 16667–16675, 1990.
166. MITCHELL, J. J., S. E. REYNOLDS, K. O. LESLIE, R. B. LOW, AND J. WOODCOCK-MITCHELL. Smooth muscle cell markers in developing rat lung. *Am. J. Respir. Cell Mol. Biol.* 3: 515–523, 1990.
167. MOISEYEVA, E. P., P. A. WELLER, N. I. ZHIDKOVA, E. B. CORBEN, B. PATEL, I. JASINSKA, V. E. KOTELIANSKY, AND D. R. CRITCHLEY. Organization of the human gene encoding the cytoskeletal protein vinculin and the sequence of the vinculin promoter. *J. Biol. Chem.* 268: 4318–4325, 1993.
168. MONICAL, P. L., G. K. OWENS, AND R. A. MURPHY. Expression of myosin regulatory light-chain isoforms and regulation of phosphorylation in smooth muscle. *Am. J. Physiol.* 264 (*Cell Physiol.* 33): C1466–C1472, 1993.
169. MOSS, N. S., AND E. P. BENDITT. Spontaneous and experimentally induced arterial lesions. I. An ultrastructural survey of the normal chicken aorta. *Lab. Invest.* 22: 166–183, 1970.
170. MOSSE, P. R., G. R. CAMPBELL, AND J. H. CAMPBELL. Smooth muscle phenotypic expression in human carotid arteries. II. Atherosclerosis-free diffuse intimal thickenings compared with the media. *Arteriosclerosis* 6: 664–669, 1986.
171. MULVANY, M. J. Abnormalities of resistance vessel structure in essential hypertension: are these important? *Clin. Exp. Pharmacol. Physiol.* 18: 13–20, 1991.
172. MULVANY, M. J. Resistance vessel structure in hypertension: growth or remodeling? *J. Cardiovasc. Pharmacol.* 22, *Suppl.* 5: S44–S47, 1993.
173. MURPHY, M. E., AND E. C. CARLSON. An ultrastructural study of developing extracellular matrix in vitelline blood vessels of the early embryo. *Am. J. Anat.* 151: 345–375, 1978.
174. MURPHY, R. Muscle cells of hollow organs. *News Physiol. Sci.* 3: 124–128, 1988.
175. MURPHY, R. A., C. M. REMBOLD, AND C. M. HAI. Contraction of smooth muscle: what is latch? *Prog. Clin. Biol. Res.* 327: 39–55, 1990.
176. MURRAY, T. R., B. E. MARSHALL, AND E. J. MACARAK. Contraction of vascular smooth muscle in cell culture. *J. Cell. Physiol.* 143: 26–38, 1990.
177. MUTHUCHAMY, M., L. PAJAK, P. HOWLES, T. DOETSCHMAN, AND D. F. WIECZOREK. Developmental analysis of tropomyosin gene expression in embryonic stem cells and mouse embryos. *Mol. Cell. Biol.* 13: 3311–3323, 1993.
178. NABESHIMA, Y., Y. NONOMURA, AND Y. FUJII-KURIYAMA. Nonmuscle and smooth muscle myosin light chain mRNAs are generated from a single gene by the tissue-specific alternative RNA splicing. *J. Biol. Chem.* 262: 10608–10612, 1987.
179. NAGAI, P. K., AND M. P. WALSH. Inhibition of smooth muscle actin-activated myosin Mg^{2+} ATPase activity by caldesmon. *J. Biol. Chem.* 259: 13656–13659, 1984.
180. NAGAI, R., M. KURO-O, P. BABIJ, AND M. PERIASAMY. Identification of two types of smooth muscle myosin heavy chain isoforms by cDNA cloning and immunoblot analysis. *J. Biol. Chem.* 264: 9734–9737, 1989.
181. NANAEV, A. K., V. P. SHIRINSKY, AND K. G. BIRUKOV. Immunofluorescent study of heterogeneity in smooth muscle cells of human fetal vessels using antibodies to myosin, desmin, and vimentin. *Cell Tissue Res.* 266: 535–540, 1991.
182. NEWCOMB, P. M., AND I. M. HERMAN. Pericyte growth and contractile phenotype: modulation by endothelial-synthesized matrix and comparison with aortic smooth muscle. *J. Cell. Physiol.* 155: 385–393, 1993.
183. NIKKARI, S. T., H. T. JARVELAINEN, T. N. WIGHT, M. FERGUSON, AND A. W. CLOWES. Smooth muscle cell expression of extracellular matrix genes after arterial injury. *Am. J. Pathol.* 144: 1348–1356, 1994.
184. NIKKARI, S. T., I. RANTALA, P. PYSTYNEN, AND T. NIKKARI. Characterization of the phenotype of smooth muscle cells in human fetal aorta on the basis of ultrastructure, immunofluorescence, and the composition of cytoskeletal and cytocontractile porteins. *Atherosclerosis* 74: 33–40, 1988.
185. NISHIDA, W., Y. KITAMI, AND K. HIWADA. cDNA cloning and mRNA expression of calponin and SM22 in rat aorta smooth muscle cells. *Gene* 130: 297–302, 1993.
186. NODEN, D. M. Embryonic origins and assembly of blood vessels. *Am. Rev. Respir. Dis.* 140: 1097–1103, 1993.

187. OBRIEN, E. R., C. E. ALPERS, D. K. STEWART, M. FERGUSON, N. TRAN, D. GORDON, E. P. BENDITT, T. HINOHARA, J. B. SIMPSON, AND S. M. SCHWARTZ. Proliferation in primary and restenotic coronary atherectomy tissue. Implications for antiproliferative therapy. *Circ. Res.* 73: 223–231, 1993.
188. OLSON, E. N. Interplay between proliferation and differentiation within the myogenic lineage. *Dev. Biol.* 154: 261–272, 1992.
189. OLSON, E. N. Regulation of muscle transcription by the MyoD family. The heart of the matter. *Circ. Res.* 72: 1–6, 1993.
190. OSTROWSKI, N. L., W. S. YOUNG, M. A. KNEPPER, AND S. J. LOLAIT. Expression of vasopressin V1a and V2 receptor messenger ribonucleic acid in the liver and kidney of embryonic, developing, and adult rats. *Endocrinology* 133: 1849–1859, 1993.
191. OWENS, G. K. Control of hypertrophic versus hyperplastic growth of vascular smooth muscle cells. *Am. J. Physiol.* 257 (*Heart Circ. Physiol.* 26): H1755–H1765, 1989.
192. OWENS, G. K., A. A. GEISTERFER, Y. W. YANG, AND A. KOMORIYA. Transforming growth factor-beta-induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells. *J. Cell Biol.* 107: 771–780, 1988.
193. OWENS, G. K., A. LOEB, D. GORDON, AND M. M. THOMPSON. Expression of smooth muscle-specific alpha-isoactin in cultured vascular smooth muscle cells: relationship between growth and cytodifferentiation. *J. Cell Biol.* 102: 343–352, 1986.
194. OWENS, G. K., P. S. RABINOVITCH, AND S. M. SCHWARTZ. Smooth muscle cell hypertrophy versus hyperplasia in hypertension. *Proc. Natl. Acad. Sci. USA* 78: 7759–7763, 1981.
195. OWENS, G. K., AND M. A. REIDY. Hyperplastic growth response of vascular smooth muscle cells following induction of acute hypertension in rats by aortic coarctation. *Circ. Res.* 57: 695–705, 1985.
196. OWENS, G. K., AND S. M. SCHWARTZ. Alterations in vascular smooth muscle mass in the spontaneously hypertensive rat. Role of cellular hypertrophy, hyperploidy and hyperplasia. *Circ. Res.* 51: 280–289, 1982.
197. OWENS, G. K., AND M. M. THOMPSON. Developmental changes in isoactin expression in rat aortic smooth muscle cells in vivo. Relationship between growth and cytodifferentiation. *J. Biol. Chem.* 261: 13373–13380, 1986.
198. PAQUET, J.-L., M. BAUDOUIN-LEGROS, G. BRUNELLE, AND P. MEYER. Angiotensin II-induced proliferation of aortic myocytes in spontaneously hypertensive rats. *J. Hypertens.* 8: 565–572, 1990.
199. PARDANAUD, L., F. YASSINE, AND F. DIETERLEN LIEVRE. Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development* 105: 473–485, 1989.
200. PARDO, J. V., J. D. SILICIANO, AND S. W. CRAIG. Vinculin is a component of an extensive network of myofibril-sarcolemma attachment regions in cardiac muscle fibers. *J. Cell Biol.* 97: 1081–1088, 1983.
201. PATEL, C. V., D. H. GORSKI, D. F. LEPAGE, J. LINCECUM, AND K. WALSH. Molecular cloning of a homeobox transcription factor from adult aortic smooth muscle. *J. Biol. Chem.* 267: 26085–26090, 1992.
202. PAULETTO, P., A. CHIAVEGATO, L. GIURIATO, M. SCATENA, E. FAGGIN, A. GRISENTI, R. SARZANI, M. V. PACI, P. D. FULGERI, A. RAPPELLI, A. C. PESSINA, AND S. SARTORE. Hyperplastic growth of aortic smooth muscle cells in renovascular hypertensive rabbits is characterized by the expansion of an immature cell phenotype. *Circ. Res.* 74: 774–788, 1994.
203. PAULY, R. R., A. PASSANITI, M. CROW, J. L. KINSELLA, N. PAPADOPOULOS, R. MONTICONE, E. G. LAKATTA, AND G. R. MARTIN. Experimental models that mimic the differentiation and dedifferentiation of vascular cells. *Circulation* 86, *Suppl.* III: III-68–III-73, 1992.
204. POLLARD, T. D. Cytoplasmic contractile proteins. *J. Cell Biol.* 91: 156–165, 1981.
205. POOLE, T. J., AND J. D. COFFIN. Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern. *J. Exp. Zool.* 251: 224–231, 1989.
206. PRICE, R. J., G. K. OWENS, AND T. C. SKALAK. Immunohistochemical identification of arteriolar development using markers of smooth muscle differentiation: evidence that capillary arterialization proceeds from terminal arterioles. *Circ. Res.* 75: 520–527, 1994.
207. RAGHOW, R. Regulation of messenger RNA turnover in eukaryotes. *Trends Biochem. Sci.* 12: 358–360, 1987.
208. RHODES, S. J., AND S. F. KONIECZNY. Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev.* 3: 2050–2061, 1989.
209. RISAU, W., P. GAUTSCHI SOVA, AND P. BOHLEN. Endothelial cell growth factors in embryonic and adult chick brain are related to human acidic fibroblast growth factor. *EMBO J.* 7: 959–962, 1988.
210. RISAU, W., AND V. LEMMON. Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. *Dev. Biol.* 125: 441–450, 1988.
211. ROBBINS, J. Gene targeting. The precise manipulation of the mammalian genome. *Circ. Res.* 73: 3–9, 1993.
212. ROSEN, E. M., I. D. GOLDBERG, H. M. SHAPIRO, S. E. LEVENSON, P. A. HALPIN, AND D. FARAGGI. Strain and site dependence of polyploidization of cultured rat smooth muscle. *J. Cell. Physiol.* 128: 337–344, 1986.
213. ROSS, R. The pathogenesis of atherosclerosis—an update. *N. Engl. J. Med.* 314: 488–500, 1986.
214. ROSS, R. Rous-Whipple Award Lecture. Atherosclerosis: a defense mechanism gone awry. *Am. J. Pathol.* 143: 987–1002, 1993.
215. ROTHMAN, A., T. J. KULIK, M. B. TAUBMAN, B. C. BERK, C. W. SMITH, AND B. NADAL GINARD. Development and characterization of a cloned rat pulmonary arterial smooth muscle cell line that maintains differentiated properties through multiple subcultures. *Circulation* 86: 1977–1986, 1992.
216. ROVNER, A. S., R. A. MURPHY, AND G. K. OWENS. Expression of smooth muscle and nonmuscle myosin heavy chains in cultured vascular smooth muscle cells. *J. Biol. Chem.* 261: 14740–14745, 1986.
217. ROVNER, A. S., M. M. THOMPSON, AND R. A. MURPHY. Two different heavy chains are found in smooth muscle myosin. *Am. J. Physiol.* 250 (*Cell Physiol.* 19): C861–C870, 1986.
218. RUBBIA, L., AND G. GABBIANI. The cytoskeleton of arterial smooth muscle cells during development, atheromatosis, and tissue culture. *J. Cardiovasc. Pharmacol.* 14, *Suppl.* 6: S9–S11, 1989.
219. RUDNICKI, M. A., P. N. SCHNEGELSBERG, R. H. STEAD, T. BRAUN, H. H. ARNOLD, AND R. JAENISCH. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* 75: 1351–1359, 1993.
220. RUIZ OPAZO, N., AND B. NADAL GINARD. Alpha-tropomyosin gene organization. Alternative splicing of duplicated isotype-specific exons accounts for the production of smooth and striated muscle isoforms. *J. Biol. Chem.* 262: 4755–4765, 1987.
221. RUZICKA, D. L., AND R. J. SCHWARTZ. Sequential activation of alpha-actin genes during avian cardiogenesis: vascular smooth muscle alpha-actin gene transcripts mark the onset of cardiomyocyte differentiation. *J. Cell Biol.* 107: 2575–2586, 1988.
222. SAINT-JEANNET, J. P., G. LEVI, J. M. GIRAULT, V. KOTELIANSKY, AND J. P. THIERY. Ventrolateral regionalization of *Xenopus laevis* mesoderm is characterized by the expression of alpha-smooth muscle actin. *Development* 115: 1165–1173, 1992.
223. SALTIS, J., A. AGROTIS, AND A. BOBIK. Age-dependent alterations in vascular smooth muscle cell responsiveness to platelet-derived growth factor in genetic hypertension. *Clin. Exp. Pharmacol. Physiol.* 20: 324–326, 1993.
224. SANTORO, I. M., AND K. WALSH. Natural and synthetic DNA elements with the CArG motif differ in expression and protein-binding properties. *Mol. Cell. Biol.* 11: 6296–6305, 1991.
225. SAPPINO, A. P., I. MASOUYE, J. H. SAURAT, AND G. GABBIANI. Smooth muscle differentiation in scleroderma fibroblastic cells. *Am. J. Pathol.* 137: 585–591, 1990.
226. SARTORE, S., M. SCATENA, A. CHIAVEGATO, E. FAGGIN, L. GIURIATO, AND P. PAULETTO. Myosin isoform expression in smooth muscle cells during physiological and pathological vascular remodeling. *J. Vasc. Res.* 31: 61–81, 1994.
227. SARTORELLI, V., M. KURABAYASHI, AND L. KEDES. Muscle-specific gene expression. A comparison of cardiac and skeletal muscle transcription strategies. *Circ. Res.* 72: 925–931, 1993.
228. SATO, Y., R. TSUBOI, R. LYONS, H. MOSES, AND D. B. RIFKIN. Characterization of the activation of latent TGF-beta by co-cultures

of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. *J. Cell Biol.* 111: 757–763, 1990.
229. SAWTELL, N. M., AND J. L. LESSARD. Cellular distribution of smooth muscle actins during mammalian embryogenesis: expression of the alpha-vascular but not the gamma-enteric isoform in differentiating striated myocytes. *J. Cell Biol.* 109: 2929–2937, 1989.
230. SCHAART, G., C. VIEBAHN, W. LANGMANN, AND F. RAMAEKERS. Desmin and titin expression in early postimplantation mouse embryos. *Development* 107: 585–596, 1989.
231. SCHAFER, B. W., AND J. C. PERRIARD. Intracellular targeting of isoproteins in muscle cytoarchitecture. *J. Cell Biol.* 106: 1161–1170, 1988.
232. SCHELL, D. N., D. DURHAM, S. S. MURPHREE, K. H. MUNTZ, AND P. W. SHAUL. Ontogeny of beta-adrenergic receptors in pulmonary arterial smooth muscle, bronchial smooth muscle, and alveolar lining cells in the rat. *Am. J. Respir. Cell Mol. Biol.* 7: 317–324, 1992.
233. SCHUBERT, D. A., A. J. HARRIS, C. E. DEVINE, AND S. HEINEMANN. Characterization of a unique muscle cell line. *J. Cell Biol.* 61: 398–413, 1974.
234. SCHWARTZ, S. M., G. R. CAMPBELL, AND J. H. CAMPBELL. Replication of smooth muscle cells in vascular disease. *Circ. Res.* 58: 427–444, 1986.
235. SCHWARTZ, S. M., L. FOY, D. F. BOWEN-POPE, AND R. ROSS. Derivation and properties of platelet-derived growth factor-independent rat smooth muscle cells. *Am. J. Pathol.* 136: 1417–1428, 1990.
236. SCHWARTZ, S. M., C. M. GAJDUSEK, AND S. C. SELDEN. Vascular wall growth control: the role of the endothelium. *Arteriosclerosis* 1: 107–161, 1981.
237. SCHWARTZ, S. M., AND M. A. REIDY. Common mechanisms of proliferation of smooth muscle in atherosclerosis and hypertension. *Hum. Pathol.* 18: 240–247, 1987.
238. SCHWARTZ, S. M., M. R. REIDY, AND A. CLOWES. Kinetics of atherosclerosis: a stem cell model. *Ann. NY Acad. Sci.* 454: 292–304, 1985.
239. SCHWARTZ, S. M., AND R. ROSS. Cellular proliferation in atherosclerosis and hypertension. *Prog. Cardiovasc. Dis.* 26: 355–372, 1984.
240. SEIDEL, C. L., C. L. WALLACE, D. K. DENNISON, AND J. C. ALLEN. Vascular myosin expression during cytokinesis,attachment, and hypertrophy. *Am. J. Physiol.* 256 (*Cell Physiol.* 25): C793–C798, 1989.
241. SHANAHAN, C. M., P. L. WEISSBERG, AND J. C. METCALFE. Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. *Circ. Res.* 73: 193–204, 1993.
242. SHAUL, P. W., R. R. MAGNESS, K. H. MUNTZ, D. DEBELTZ, AND L. M. BUJA. Alpha 1-adrenergic receptors in pulmonary and systemic vascular smooth muscle. Alterations with development and pregnancy. *Circ. Res.* 67: 1193–1200, 1990.
243. SHIMIZU, R. T., R. S. BLANK, R. JERVIS, S. C. LAWRENZ-SMITH, AND G. K. OWENS. The smooth muscle actin gene promoter is differentially regulated in smooth muscle versus non-smooth muscle cells. *J. Biol. Chem.* 270: 7631–7643, 1995.
244. SHYU, A., M. E. GREENBERG, AND J. G. BELASCO. The c-*fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways. *Genes Dev.* 3: 60–72, 1989.
245. SILICIANO, J. D., AND S. W. CRAIG. Properties of smooth muscle meta-vinculin. *J. Cell Biol.* 104: 473–482, 1987.
246. SIMPSON, D. G., W. CARVER, T. K. BORG, AND L. TERRACIO. Role of mechanical stimulation in the establishment and maintenance of muscle cell differentiation. *Int. Rev. Cytol.* 150: 69–94, 1994.
247. SJOLUND, M., U. HEDIN, T. SEJERSEN, C. H. HELDIN, AND J. THYBERG. Arterial smooth muscle cells express platelet-derived growth factor (PDGF) A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state-dependent manner. *J. Cell Biol.* 106: 403–413, 1988.
248. SKALLI, O., P. ROPRAZ, A. TRZECIAK, G. BENZONANA, D. GILLESSEN, AND G. GABBIANI. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J. Cell Biol.* 103: 2787–2796, 1986.
249. SMALL, J. V., AND A. SOBIESZEK. Studies on the function and composition of the 10-nm (100-A) filaments of vertebrate smooth muscle. *J. Cell Sci.* 23: 243–268, 1977.
250. SMITH, J. B. Angiotensin-receptor signaling in cultured vascular smooth muscle cells. *Am. J. Physiol.* 250 (*Renal Fluid Electrolyte Physiol.* 19): F759–F769, 1986.
251. SOBUE, K., Y. MURAMOTO, M. FUJITA, AND S. KAKIUCHI. Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. *Proc. Natl. Acad. Sci. USA* 78: 5652–5655, 1981.
252. SOBUE, K., AND J. R. SELLERS. Caldesmon, a novel regulatory protein in smooth muscle and nonmuscle actomyosin systems. *J. Biol. Chem.* 266: 12115–12118, 1991.
253. SOMLYO, A. P. Myosin isoforms in smooth muscle: how may they affect function and structure. *J. Muscle Res. Cell Motil.* 14: 557–563, 1993.
254. STERPETTI, A. V., A. CUCINA, L. S. D'ANGELO, B. CARDILLO, AND A. CAVALLARO. Shear stress modulates the proliferation rate, protein synthesis, and mitogenic activity of arterial smooth muscle cells. *Surgery* 113: 691–699, 1993.
255. ST. LEZIN, E., L. SIMONET, M. PRAVENEC, AND T. W. KURTZ. Hypertensive strains and normotensive "control" strains. How closely are they related? *Hypertension Dallas* 19: 419–424, 1992.
256. STOFLET, E. S., L. J. SCHMIDT, P. K. ELDER, G. M. KORF, D. N. FOSTER, A. R. STRAUCH, AND M. J. GETZ. Activation of a muscle-specific actin gene promoter in serum-stimulated fibroblasts. *Mol. Biol. Cell* 3: 1073–1083, 1992.
257. STOUFFER, G. A., AND G. K. OWENS. TGF-beta promotes proliferation of cultured SMC via both PDGF-AA-dependent and PDGF-independent mechanisms. *J. Clin. Invest.* 93: 2048–2055, 1994.
258. STRASSER, P., M. GIMONA, H. MOESSLER, M. HERZOG, AND J. V. SMALL. Mammalian calponin: identification and expression of genetic variants. *FEBS Lett.* 330: 13–18, 1993.
259. STRAUCH, A. R., B. MIN, J. C. REESER, M. D. BERMAN, H. R. MILLER, AND D. N. FOSTER. Cellular and molecular control of vascular smooth muscle alpha-actin gene expression during BC_3H1 myogenic cell differentiation. *Prog. Clin. Biol. Res.* 327: 583–589, 1990.
260. SUGI, Y., AND J. LOUGH. Onset of expression and regional deposition of alpha-smooth and sarcomeric actin during avian heart development. *Dev. Dynamics* 193: 116–124, 1992.
261. SUNDELL, C., AND R. SINGER. Requirements of microfilaments for actin mRNA sorting. *Science Wash. DC* 253: 1275–1278, 1991.
262. TAGAMI, M., Y. NARA, A. KUBOTA, T. SUNAGA, H. MAEZAWA, H. FUJINO, AND Y. YAMORI. Morphological and functional differentiation of cultured vascular smooth muscle cells. *Cell Tissue Res.* 245: 261–266, 1986.
263. TAKAHASHI, K., K. HIWADA, AND T. KOKUBU. Occurrence of antigizzard P34K antibody cross-reactive components in bovine smooth muscles and non-smooth muscle tissues. *Life Sci.* 41: 291–296, 1987.
264. TAKAHASHI, K., AND B. NADAL-GINARD. Molecular cloning and sequence analysis of smooth muscle calponin. *J. Biol. Chem.* 266: 13284–13288, 1991.
265. TAKANO-OHMURO, H., T. OBINATA, T. MIKAWA, AND T. MASAKI. Changes in myosin isozymes during development of chicken gizzard muscle. *J. Biochem.* 93: 903–908, 1983.
266. TAKEUCHI, K., K. TAKAHASHI, M. ABE, W. NISHIDA, K. HIWADA, T. NABEYA, AND K. MARUYAMA. Co-localization of immunoreactive forms of calponin with actin cytoskeleton in platelets, fibroblasts, and vascular smooth muscle. *J. Biochem.* 109: 311–316, 1991.
267. TAPSCOTT, S. J., R. L. DAVIS, M. J. THAYER, P. F. CHENG, H. WEINTRAUB, AND A. B. LASSAR. MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science Wash. DC* 242: 405–411, 1988.
268. TAUBMAN, M. B., J. W. GRANT, AND B. NADAL GINARD. Cloning and characterization of mammalian myosin regulatory light chain (RLC) cDNA: the RLC gene is expressed in smooth, sarcomeric, and nonmuscle tissues. *J. Cell Biol.* 104: 1505–1513, 1987.
269. TAUBMAN, M. B., C. W. SMITH, S. IZUMO, J. W. GRANT, T. ENDO, A. ANDREADIS, AND B. NADAL GINARD. The expression of sarcomeric muscle-specific contractile protein genes in BC_3H1 cells: BC_3H1 cells resemble skeletal myoblasts that are defective for com-

mitment to terminal differentiation. *J. Cell Biol.* 108: 1799–1806, 1989.

270. THYBERG, J., U. HEDIN, M. SJOLUND, L. PALMBERG, AND B. BOTTGER. Regulation of differentiated properties and proliferation of arterial smooth muscle cells. *Arteriosclerosis* 10: 966–990, 1990.
271. THYBERG, J., L. PALMBERG, J. NILSSON, T. KSIAZEK, AND M. SJOLUND. Phenotypic modulation in primary cultures of arterial smooth muscle cells: on the role of platelet-derived growth factor. *Differentiation* 25: 156–167, 1983.
272. TONTONOZ, P., J. B. KIM, R. A. GRAVES, AND B. M. SPIEGELMAN. ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Mol. Cell. Biol.* 13: 4753–4759, 1993.
273. TURLA, M. B., M. M. THOMPSON, M. H. CORJAY, AND G. K. OWENS. Mechanisms of angiotensin II- and arginine vasopressin-induced increases in protein synthesis and content in cultured rat aortic smooth muscle cells. Evidence for selective increases in smooth muscle isoactin expression. *Circ. Res.* 68: 288–299, 1991.
274. TUTTLE, J. B., AND D. J. CREEDON. Pharmacology of nerve growth factor output by target cells. *Ann. NY Acad. Sci.* 692: 273–276, 1993.
275. UEKI, N., K. SOBUE, K. KANDA, T. HADA, AND K. HIGASHINO. Expression of high and low molecular weight caldesmons during phenotypic modulation of smooth muscle cells. *Proc. Natl. Acad. Sci. USA* 84: 9049–9053, 1987.
276. VANDEKERCKHOVE, J., AND K. WEBER. The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. *Differentiation* 14: 123–133, 1979.
277. VANDENBURGH, H. H. Motion into mass: how does tension stimulate muscle growth? *Med. Sci. Sports Exercise* 19, *Suppl.* 5: S142–S149, 1987.
278. VAN NECK, J. W., J. J. MEDINA, C. ONNEKINK, P. F. VAN DER VEN, H. P. BLOEMERS, AND S. M. SCHWARTZ. Basic fibroblast growth factor has a differential effect on MyoD conversion of cultured aortic smooth muscle cells from newborn and adult rats. *Am. J. Pathol.* 143: 269–282, 1993.
279. VERNON, S. M., I. J. SAREMBOCK, AND G. K. OWENS. Thrombin-induced proliferation in cultured smooth muscle cells is not associated with downregulation of smooth muscle α-actin gene expression (Abstract). *Circulation* 88, *Suppl.* I: I-189, 1993.
280. VERNON, S. M., M. M. THOMPSON, AND G. K. OWENS. Endothelial conditioned media downregulates expression of smooth muscle specific contractile proteins in cultured rat aortic smooth muscle cells (Abstract). *Circulation* 86, *Suppl.* I: I-761, 1992.
281. VIRAGH, S., E. SZABO, AND C. E. CHALLICE. Formation of the primitive myo- and endocardial tubes in the chicken embryo. *J. Mol. Cell. Cardiol.* 21: 123–137, 1989.
282. VISWANATHAN, M., K. TSUTSUMI, F. M. CORREA, AND J. M. SAAVEDRA. Changes in expression of angiotensin receptor subtypes in the rat aorta during development. *Biochem. Biophys. Res. Commun.* 179: 1361–1367, 1991.
283. WALKER, L. N., D. F. BOWEN POPE, R. ROSS, AND M. A. REIDY. Production of platelet-derived growth factor-like molecules by cultured arterial smooth muscle cells accompanies proliferation after arterial injury. *Proc. Natl. Acad. Sci. USA* 83: 7311–7315, 1986.
284. WEINTRAUB, H. The MyoD family and myogenesis: redundancy, networks. and thresholds. *Cell* 75: 1241–1244, 1993.
285. WEINTRAUB, H., R. DAVIS, S. TAPSCOTT, M. THAYER, M. KRAUSE, R. BENEZRA, T. K. BLACKWELL, D. TURNER, R. RUPP, AND S. HOLLENBERG. The myoD gene family: nodal point during specification of the muscle cell lineage. *Science Wash. DC* 251: 761–766, 1991.
286. WHITE, S., A. F. MARTIN, AND M. PERIASAMY. Identification of a novel smooth muscle myosin heavy chain cDNA: isoform diversity in the S1 head region. *Am. J. Physiol.* 264 (*Cell Physiol.* 33): C1252–C1258, 1993.
287. WIECZOREK, D. F., C. W. SMITH, AND B. NADAL GINARD. The rat alpha-tropomyosin gene generates a minimum of six different mRNAs coding for striated, smooth, and nonmuscle isoforms by alternative splicing. *Mol. Cell. Biol.* 8: 679–694, 1988.
288. WILSON, E., Q. MAI, K. SUDHIR, R. H. WEISS, AND H. E. IVES. Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. *J. Cell Biol.* 123: 741–747, 1993.
289. WINDER, S. J., C. SUTHERLAND, AND M. P. WALSH. Biochemical and functional characterization of smooth muscle calponin. *Adv. Exp. Med. Biol.* 304: 37–51, 1991.
290. WINDER, S. J., C. SUTHERLAND, AND M. P. WALSH. A comparison of the effects of calponin on smooth and skeletal muscle actomyosin systems in the presence and absence of caldesmon. *Biochem. J.* 288: 733–739, 1992.
291. WOODCOCK-MITCHELL, J., J. J. MITCHELL, R. B. LOW, M. KIENY, P. SENGEL, L. RUBBIA, O. SKALLI, B. JACKSON, AND G. GABBIANI. Alpha-smooth muscle actin is transiently expressed in embryonic rat cardiac and skeletal muscles. *Differentiation* 39: 161–166, 1988.
292. WRIGHT, W. E., D. A. SASSOON, AND V. K. LIN. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* 56: 607–617, 1989.
293. ZANELLATO, A. M. C., A. C. BORRIONE, M. TONELLO, G. SCANNAPIECO, P. PAULETTO, AND S. SARTORE. Myosin isoform expression in smooth muscle cell heterogeneity in normal and atherosclerotic rabbit aorta. *Arteriosclerosis* 10: 996–1009, 1990.
294. ZUCKER, M., AND P. STEIGLER. Optimal computer folding of large RNA sequences using thermodynamics and auxillary information. *Nucleic Acids Res.* 9: 133–148, 1981.

HOME
PREMIUM SERVICES
M-WCollegiate.com
M-WUnabridged.com
Britannica.com
Multi-User Licenses
DOWNLOADS
WORD OF THE DAY
WORD GAMES
WORD FOR THE WISE
ONLINE STORE
HELP
Merriam-Webster Info
Company Information

Merriam-Webster Online Dictionary

Thesaurus

One entry found for **inhibit**.

Main Entry: **in·hib·it**
Pronunciation: `in-'hi-b&t`
Function: *verb*
Etymology: Middle English, from Latin *inhibitus*, past participle of *inhibEre*, from *in-* [2]in- + *habEre* to have -- more at HABIT
transitive senses
1 : to prohibit from doing something
2 a : to hold in check **:** RESTRAIN **b :** to discourage from free or spontaneous activity especially through the operation of inner psychological impediments or of social controls
intransitive senses **:** to cause inhibition
synonym see FORBID
- **in·hib·i·tive** /-b&-tiv/ *adjective*
- **in·hib·i·to·ry** /-b&-"tOr-E, -"tor-/ *adjective*

For **More Information on "inhibit" go to Britannica.com**

Get the **Top 10 Search Results for "inhibit"**

Merriam-Webster Online Dictionary

Thesaurus

One entry found for **normalize**.

Main Entry: **nor·mal·ize**
Pronunciation: 'nor-m&-"lIz
Function: *transitive verb*
Inflected Form(s): **-ized**; **-iz·ing**
1 : to make conform to or reduce to a norm or standard
2 : to make normal (as by a transformation of variables)
3 : to bring or restore (as relations between countries) to a normal condition
- **nor·mal·iz·able** /-"lI-z&-b&l/ *adjective*
- **nor·mal·i·za·tion** /"nor-m&-l&-'zA-sh&n/ *noun*

For **More Information on "normalize" go to Britannica.com**

Get the **Top 10 Search Results for "normalize"**

Pronunciation Symbols

Chloride ion currents contribute functionally to norepinephrine-induced vascular contraction

F

FRED S. LAMB AND THOMAS J. BARNA
Department of Pediatrics, University of Iowa, Iowa City, Iowa, 52242

Lamb, Fred S., and Thomas J. Barna. Chloride ion currents contribute functionally to norepinephrine-induced vascular contraction. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H151–H160, 1998.—Norepinephrine (NE) increases Cl^- efflux from vascular smooth muscle (VSM) cells. An increase in Cl^- conductance produces membrane depolarization. We hypothesized that if Cl^- currents are important for agonist-induced depolarization, then interfering with cellular Cl^- handling should alter contractility. Isometric contraction of rat aortic rings was studied in a bicarbonate buffer. Substitution of extracellular Cl^- with 130 mM methanesulfonate (MS; 8 mM Cl^-) did not cause contraction. NE- and serotonin-induced contractions were potentiated in this low-Cl^- buffer, whereas responses to K^+, BAY K 8644, or NE in the absence of Ca^{2+} were unaltered. Substitution of Cl^- with I^- or Br^- suppressed responses to NE. Inhibition of Cl^- transport with bumetanide (10^{-5} M) or bicarbonate-free conditions (10 mM HEPES) inhibited NE- but not KCl-induced contraction. The Cl^--channel blockers DIDS (10^{-3} M), anthracene-9-carboxylic acid (10^{-3} M), and niflumic acid (10^{-5} M) all inhibited NE-induced contraction, whereas tamoxifen (10^{-5} M) did not. Finally, disruption of sarcoplasmic reticular function with cyclopiazonic acid (10^{-7} M) or ryanodine (10^{-5} M) prevented the increase in the peak response to NE produced by low-Cl^- buffer. We conclude that a Cl^- current with a permeability sequence of $I^- > Br^- > Cl^- >$ MS is critical to agonist-induced contraction of VSM.

chloride channels; vascular smooth muscle; sodium-potassium-chloride cotransport; chloride/bicarbonate exchange; sarcoplasmic reticulum

UNDER MOST CIRCUMSTANCES vascular smooth muscle (VSM) tone and contractility are tightly coupled to membrane potential (V_m) (29). This relationship is maintained by the dependence of contraction on the influx of Ca^{2+} through voltage-dependent, primarily L-type Ca^{2+} channels. Numerous studies have attempted to define the role of various K^+ conductances (delayed rectifier, Ca^{2+} activated, ATP dependent) in the control of resting V_m, agonist-induced depolarization, and vasodilatation (4, 11, 18, 40). Conversely, although Cl^- channels are present in VSM (23, 27) and are activated by agonists (24, 32), there is minimal functional evidence defining the contribution of these events to contractile responses.

The manner in which VSM cells handle Cl^- sets up an ideal system for producing and maintaining depolarization. VSM cells accumulate Cl^- intracellularly through several processes (9, 13, 14), including Na^+-K^+-$2Cl^-$ cotransport, Cl^-/HCO_3^- exchange, and a third component, possibly an ATP-dependent transporter (16). Whereas resting V_m in VSM ranges from approximately −45 to −65 mV, estimates of the Cl^- equilibrium potential [$E_{Cl} = -60 \log$ (extracellular Cl^- concentration/intracellular Cl^- concentration)], measured using either radiolabeled Cl^- flux (25, 38) or ion-selective microelectrodes (16), range between −11 and −50 mV. In any given vascular tissue, E_{Cl} has always been measured to be roughly 15–30 mV more positive than V_m. Depolarization of the precise magnitude induced by vasoconstrictors can therefore be readily produced by an increase in Cl^- conductance.

Estimates of relative permeabilities at rest for Cl^- and K^+ have varied widely from 0.09 in rat femoral artery (10) to 0.82 in rat portal vein (38); however, this ratio may change dramatically after exposure to a contractile agonist. Adrenergic stimulation has frequently been shown to increase total membrane conductance [rabbit carotid artery (28) and pulmonary artery (7), guinea pig mesenteric artery (3) and pulmonary artery (5)] consistent with the activation of a Cl^- current (decrease in membrane resistance). Occasionally, total membrane conductance has been found to decrease [guinea pig ear artery (22), pulmonary artery (35)]. These contrasting results may be due to functional differences between tissues. Alternatively, the interpretation of these results may be complicated by the syncytial nature of VSM. Changes in membrane resistance may be obscured or magnified by changes in the electrical coupling between cells.

Radiolabeled ion flux data are less dependent on cell-cell coupling. Norepinephrine (NE) dramatically increases the efflux of both $^{42}K^+$ and $^{36}Cl^-$ from rabbit pulmonary artery (7, 34). The computed increase in permeability for Cl^- exceeds that for K^+ (21). Because depolarization moves V_m closer to E_{Cl} (diminishing the driving force for Cl^- movement), it is difficult to explain an increase in Cl^- efflux unless there is a significant increase in Cl^- conductance. Conversely, the observed increase in K^+ efflux is difficult to reconcile with a mechanism of depolarization that is driven primarily by the alternative proposed mechanism, a decrease in K^+ conductance.

Agonist-induced Cl^- currents have now been characterized in a number of vascular tissues. NE activates a Cl^- current in cells from portal vein (6, 32), mesenteric vein (36), and ear artery (2). Endothelin elicits a similar current in coronary, aortic, and mesenteric VSM cells (24, 37), as does vasopressin in cultured aortic cells (17, 37). These Cl^- currents are Ca^{2+} dependent ($I_{Cl,Ca}$) (27, 31) and appear to be activated initially by agonist-induced release of intracellular Ca^{2+} stores. The extent to which Ca^{2+} entry from extracellular sources can sustain activation of these channels is unknown. At the single-channel level they appear to have a low conductance of 1–2 pS (23). Another Cl^- channel that has been characterized in VSM at the single-channel level is a large conductance channel (340 pS) that is activated by

protein kinase C inhibitors (and is therefore likely inhibited by protein kinase C-dependent phosphorylation) in cell-attached patches (33). VSM also contains a typical volume-activated Cl^- current (30).

Despite the abundance of evidence documenting the existence of agonist-induced Cl^- currents, the degree to which the resulting depolarization contributes to contraction is not known. We have employed a strategy involving *1*) alteration of E_{Cl}, *2*) inhibition of Cl^- transport, and *3*) block of Cl^- channels to obtain conclusive functional evidence that Cl^- currents are critical to agonist-induced activation of vascular smooth muscle.

METHODS

Adult male Sprague-Dawley rats (250–300 g) were obtained from Harlan Sprague Dawley. The animals were killed by exposure to 100% CO_2 for 5 min, followed by cervical dislocation. Thoracic aortas were removed, cleaned of adherent connective tissue, and cut into 6-mm rings. The endothelium was left intact, and the rings were mounted in individual 10-ml isolated organ chambers (Radnoti Glass) using stainless steel triangles and were connected to an isometric force transducer (Kent Scientific) by 32-gauge stainless steel wire. Contractile responses were recorded with an eight-channel MacLab 8E and stored on a Power Macintosh 7200 computer. Passive stretch was set at 2.5 g, and the rings were allowed to equilibrate in physiological salt solution (PSS) at 37°C for 120 min before the start of experimentation. PSS was aerated with a mixture of 95% O_2-5% CO_2; the composition was as follows (in mM): 130 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4 \cdot 7H_2O$, 14.9 $NaHCO_3$, 1.6 $CaCl_2 \cdot H_2O$, 5.5 dextrose, and 0.03 $CaNa_2$-EDTA 0.03 (pH 7.30). The standard low-Cl^- buffer was prepared by substituting NaCl with 130 mM NaOH and titrating the pH of the buffer to 7.30 with methanesulfonic acid while the solution was being aerated. The measured osmolality of the low-Cl^- buffer was 292 mosmol/kg compared with 293 mosmol/kg for the control buffer (5500 vapor pressure osmometer). For the concentration-response curve to extracellular Cl^- (Fig. 4), the various Cl^- concentrations were achieved by mixing standard PSS with low-Cl^- buffer in fixed ratios. Low-Cl^- buffers substituted with Br^- or I^- were made by replacing 130 mM NaCl completely with NaBr or NaI. HEPES buffer was made by substituting 10 mM HEPES for bicarbonate and titrating the pH of the solution to 7.30 using 1 M NaOH. When both HEPES and bicarbonate were used, pH was once again 7.30.

In all experiments in which extracellular Cl^- was altered suddenly, the low-Cl^- buffer was prewarmed and preaerated in a 37°C constant temperature bath before the solution was poured directly into the drained organ chamber. This was done to avoid depletion of intracellular Cl^- before exposure to the contractile agent. Control responses for these experiments were also recorded after the agonist-containing control buffer was poured into the bath.

At the beginning of each experiment a contractile response to 120 mM KCl was recorded. Subsequent contractile responses are normalized by expression as a percentage of this initial maximal response to KCl. All agonist-induced contractions were recorded for 20 min. When KCl was used to induce contraction, it was added directly to the buffer from a 1 M stock without adjusting for changes in tonicity. When K^+ was used as an agonist in low-Cl^- conditions, it was added from a 1 M stock of K-methanesulfonate. This stock was prepared by titrating the pH of 1 M KOH to 7.3 with methanesulfonic acid. Ca^{2+}-free PSS was prepared by omission of $CaCl_2$ without the use of an additional chelating agent. The bath was washed three times in this buffer over an ~5 min period before addition of the agonist.

There are two readily recognized phases to VSM contraction. The initial contractile response has been attributed to direct activation of the contractile proteins by Ca^{2+} released from the sarcoplasmic reticulum (SR). Our hypothesis maintains that a second, important function of this Ca^{2+} pool is to activate a depolarizing Cl^- current. This depolarization results in the influx of extracellular Ca^{2+}, which is responsible for the second, or maintained, phase of contraction. To make a functional assessment of both phases of contraction, the contractions are measured at both an early and a late time point. Lower concentrations of agonist [~20% effective dose (ED_{20})] generally produce a clear early peak of contraction (~3 min) that exceeds the tension measured once contraction has stabilized. These data (see Figs. 1, 2, 4, 5, and 10) are displayed as paired bars, peak contraction (highest tension recorded at ≤3 min), and tension at the 20-min time point. In experiments in which higher concentrations of agonist are used [~80% effective dose (ED_{80})] to study inhibition of contraction (see Figs. 6–9), there is generally no early peak seen. Tension increases rapidly over the first 5 min and then more slowly until a stable level is achieved at ~20 min. These data are displayed as tension at the 5- and 20-min time points.

NE, serotonin (5-HT), DIDS, and ryanodine were dissolved directly into aqueous solution. BAY K 8644 and bumetanide were prepared as stock solutions in ethanol, whereas ethacrynic acid, tamoxifen, niflumic acid, anthracene-9-carboxylic acid (A-9-C), and cyclopiazonic acid (CPA) were prepared as stock solutions in DMSO. The drugs were added to the buffer at no greater than a 1,000:1 dilution, yielding a final solvent concentration of 0.1%. No individual ring was used for more than a single pharmacological intervention (drug or combination of drugs) to avoid potential effects of incomplete washout. All drugs and salts for the preparation of PSS were obtained from Sigma Chemical with the exception of BAY K 8644 (Calbiochem).

Data are displayed as means ± SE, and n represents the number of rats in each group. Statistical analysis of group differences was performed using Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Contractile responses to NE and 5-HT but not to KCl are potentiated in low-Cl^- buffer. The concentrations of these contractile agents were titrated in each individual ring to achieve a control response that was ~20% of the response to 120 mM KCl. Figure 1*A* shows the effect of low-Cl^- buffer on the peak and maintained phases of contraction in response to these agents. The potentiating effect of low-Cl^- is more dramatic at the 3-min time point but remains significant after 20 min. The force recording shown in Fig. 1*B* demonstrates the typical pattern of phasic contractions that occur during the initial phase of contraction in low-Cl^- buffer. Mean concentrations used were 2×10^{-8} M for NE ($n = 9$), 1.6×10^{-6} M for 5-HT ($n = 6$), and 18 mM for K^+ ($n = 5$). Although both NE and 5-HT elicit SR Ca^{2+} release and can activate $I_{Cl,Ca}$, K^+ cannot. All of these experiments (including control responses) were performed by exposing the rings to NE and to the altered extracellular Cl^- concentration at the same moment by

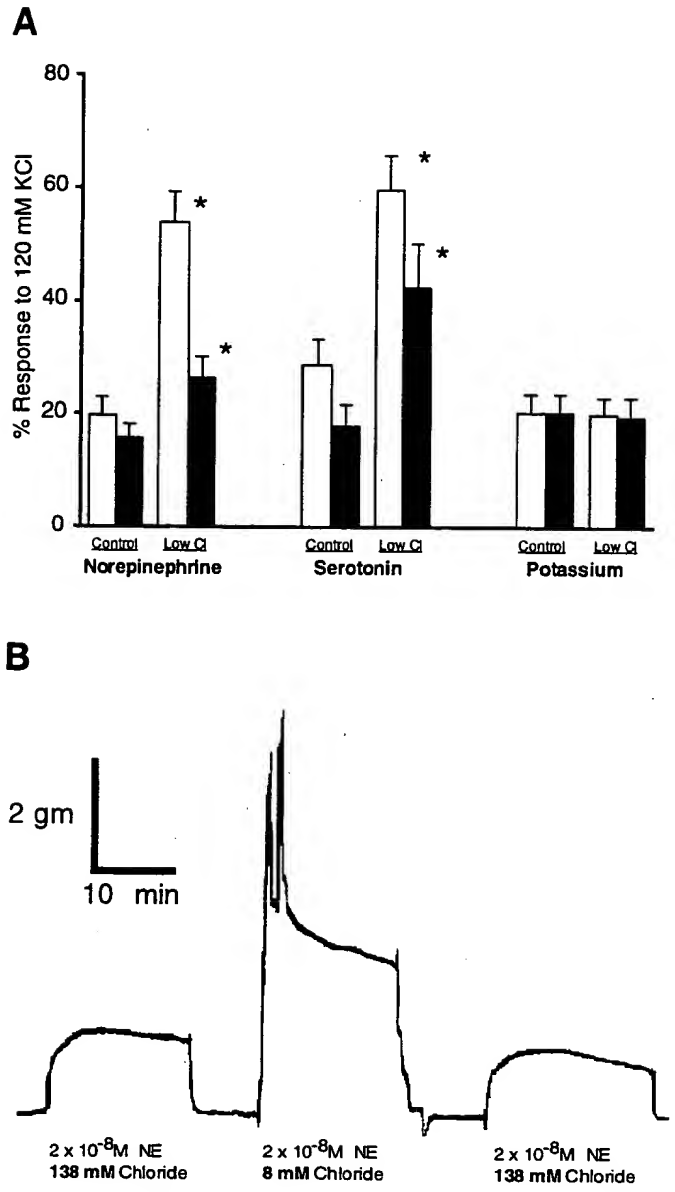


Fig. 1. Contractile responses to norepinephrine (NE) and serotonin but not to K^+ are potentiated by low-Cl^- buffer. *A*: both peak (3 min, open bars) and maintained (20 min, solid bars) contractile responses to NE and serotonin are significantly larger than control in low-Cl^- buffer while responses to K^+ are unaltered. $*P < 0.05$. *B*: typical response of an aortic ring to NE in control (138 mM) and low-Cl^- (8 mM) buffer. The change in Cl^- concentration and addition of agonist are accomplished simultaneously by pouring on prewarmed and preaerated buffer containing the drug at the desired concentration. A follow-up control response demonstrates that the effect of low-Cl^- buffer is completely reversible.

rapidly draining the vessel chamber and replacing the buffer with prewarmed, premixed low-Cl^- buffer. Low-Cl^- buffer does not cause any contractile response by itself (data not shown).

The results from Fig. 1 suggest that Cl^- current contributes more to agonist-induced activation of VSM during the initial phase of contraction. Alternatively, the potentiating effect of low-Cl^- buffer may diminish with time as intracellular Cl^- falls and cannot be readily replenished. To distinguish between these two possibilities, rings were first contracted in normal 138 mM Cl^- buffer with ~ED_{20} NE and allowed to reach a stable, maintained level of tension (20 min). The buffer was then rapidly exchanged in an identical manner to that used before and was replaced with buffer containing the same concentration of NE and either 138 or 8 mM Cl^- [substituted with methanesulfonate (MS)]. The results are shown in Fig. 2. The contractile response is again dramatically potentiated by low-Cl^- buffer, and this effect diminishes over 5–10 min; however, tension remains significantly greater than that of the control 20 min later. These results suggest that Cl^- channels remain open during the sustained phase of contraction and that intracellular Cl^- is rapidly depleted in low-Cl^- buffer.

BAY K 8644 (5×10^{-5} M, $n = 5$), a direct Ca^{2+}-channel activator that causes Ca^{2+} influx, produced a transient contractile response in normal Ca^{2+} buffer. This response was unaltered by low-Cl^- buffer (Fig. 3). The activity of BAY K 8644 is voltage dependent, and one might expect a larger response if low-Cl^- buffer produced a significant depolarization. However, BAY K 8644 does not cause release of SR Ca^{2+} and may not produce sufficient elevation of intracellular Ca^{2+} to

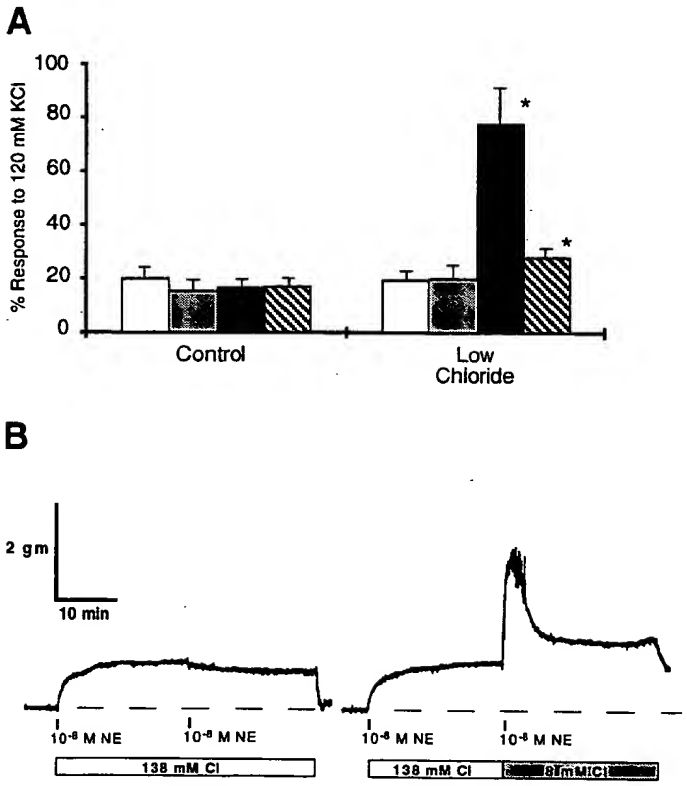


Fig. 2. Low-Cl^- buffer also potentiates NE-induced contraction during maintained phase of contraction. *A*: there is no significant change in tension when bath is drained after 20 min of contraction and replenished with fresh buffer with the same concentration of NE (control). If extracellular Cl^- is lowered when buffer is changed [8 mM Cl^- substituted with methanesulfonate (MS)], the contractile response is potentiated (low Cl^-). Open bars, first initial peak response to NE; shaded bars, first maintained response to NE; solid bars, second initial peak response to NE (fresh buffer); hatched bars, second maintained response to NE (fresh buffer). $*P < 0.05$ compared with same time point during control protocol. *B*: typical recording of a single ring showing both control (*left*) and low-Cl^- (*right*) responses.

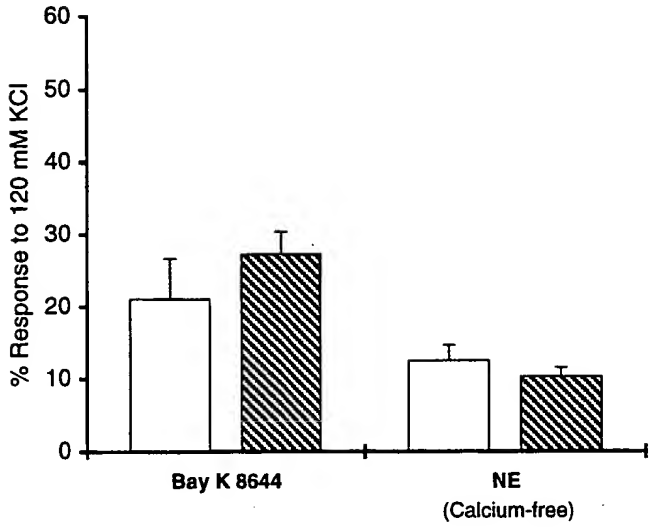


Fig. 3. BAY K 8644 in normal Ca^{2+} buffer and NE (10^{-8} M) in nominally Ca^{2+}-free buffer both produce transient contractile responses (open bars). When these responses are repeated in low-Cl^- buffer (hatched bars), there is no change in sizes of responses.

activate $I_{Cl,Ca}$. NE (10^{-8} M, $n = 6$) in the absence of extracellular Ca^{2+} produced a similar transient contractile response due to the release of intracellular Ca^{2+} stores. Again, this contraction was not effected by low-Cl^- conditions (Fig. 3). Even if a Cl^- current was activated by NE, in the absence of extracellular Ca^{2+} no Ca^{2+} influx can result from depolarization regardless of the magnitude.

The potentiating effect of low-Cl^- buffer on NE-induced contractions is dependent on the degree to which the extracellular Cl^- concentration is lowered. Figure 4 represents a concentration-response curve to lowering extracellular Cl^- (from 138 to 8 mM) using the same dose of NE at all Cl^- concentrations ($n = 4$). The peak of contraction is significantly potentiated in 41 and in 8 mM Cl^-, whereas the maintained response is enhanced only in 8 mM Cl^-.

Substitution of Cl^- with MS should make E_{Cl} more positive due to its very low permeability (no inward MS current). Substitution with an anion having a significant ability to permeate the VSM anion channels would allow inward movement down their concentration gradients (infinitely large at the instant of substitution). This movement will contribute to V_m and would be predicted to produce hyperpolarization. Figure 5 compares the effect on the peak contractile response to NE when MS ($n = 5$), Br^- ($n = 5$), or I^- ($n = 5$) are used to replace Cl^-. Once again in these tissues, Cl^- substitution with MS resulted in a significant potentiation of the peak NE-induced contraction. However, Br^- and I^- significantly suppressed the contractile response to NE, with I^- producing a more profound effect than Br^-.

Inhibitors of Cl^- transport suppress contractile responses to NE (Fig. 6). Rings were first contracted with a dose of NE titrated to produce an ~ED_{80} response (mean NE dose 4.9×10^{-8} M). After the control response was obtained, the rings were incubated for 20 min in either PSS alone (time control, $n = 4$), bumetanide (10^{-5} M, $n = 5$), 10 mM HEPES (0 mM HCO_3^-, $n = 5$), ethacrynic acid (10^{-5} M, $n = 5$), bumetanide + HEPES ($n = 4$), ethacrynic acid + HEPES ($n = 5$), bumetanide + ethacrynic acid ($n = 6$), or bumetanide + HEPES + ethacrynic acid ($n = 6$). Time alone had no effect on the magnitude of the response to NE, because the second response to NE was unchanged from control. Both bumetanide and HEPES buffer significantly suppressed NE-induced contraction, whereas ethacrynic acid produced very mild inhibition at the 5-min time point only. Bumetanide and HEPES together produced a greater inhibitory effect than either agent alone, and this combination produced as much inhibition of contraction as all three agents together.

Figure 7 demonstrates that it is the absence of bicarbonate, not the presence of HEPES, that sup-

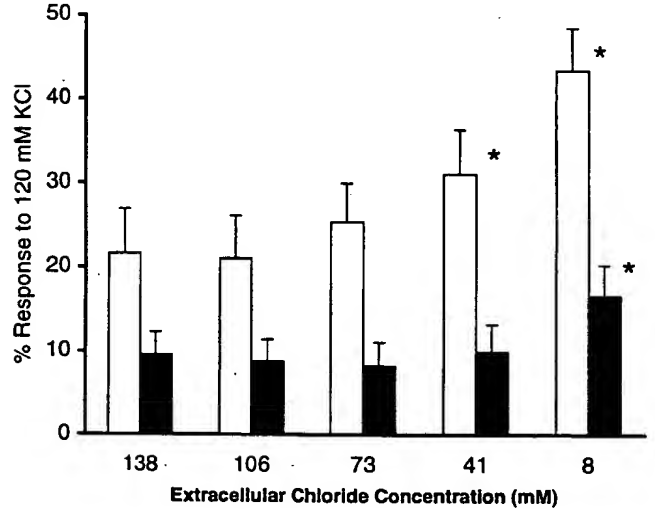


Fig. 4. Effect of lowering extracellular Cl^- is concentration dependent. Peak responses (open bars) are significantly augmented when extracellular Cl^- is lowered to 41 or 8 mM. Maintained responses (20 min, solid bars) are larger only when Cl^- concentration is lowered to 8 mM. *$P < 0.05$ compared with response in normal, 138 mM Cl^-.

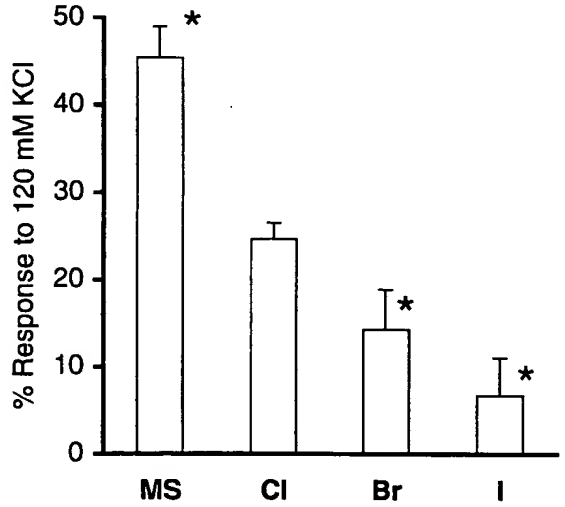


Fig. 5. Size of peak response to NE is dependent on the anion used to substitute for Cl^-. Substitution with MS for Cl^- results in potentiation of contraction. Substitution with Br^- or I^- causes suppression of initial peak response. *$P < 0.05$ compared with Cl^-.

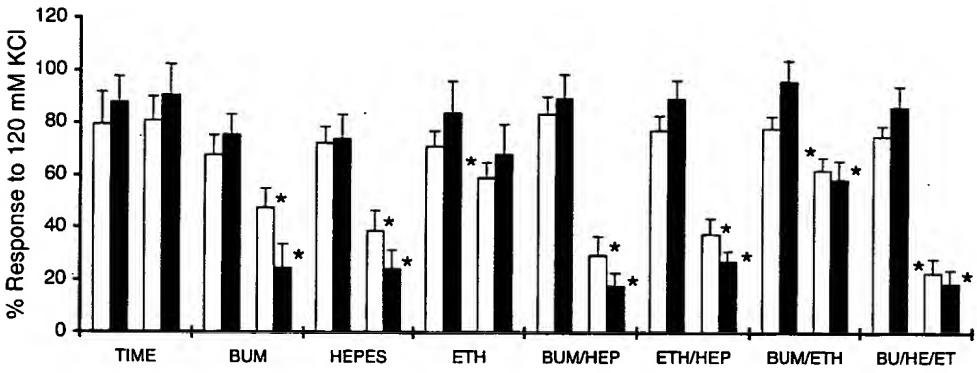


Fig. 6. NE-induced contractions are inhibited by Cl^- transport inhibitors. Control responses to NE were obtained that were ~80% of response to 120 mM KCl (ED_{80}; pairs of bars at *left* for each labeled intervention). In contrast to responses at ~20% of response to 120 mM KCl (ED_{20}), responses of this magnitude fail to show a consistent initial peak tension, and therefore force is displayed as the highest tension in the first 5 min, generally as tension at the 5-min time point (open bars), and as tension at the 20-min time point (solid bars). Control contractions were of similar magnitude in all groups. Repeat responses were obtained following a 20-min incubation in physiological salt solution only (Time, $n = 4$), 10^{-5} M bumetanide (Bum, $n = 5$), 10 mM HEPES buffer without bicarbonate (HEPES, $n = 5$), 10^{-5} M ethacrynic acid (Eth, $n = 5$), or a combination of interventions [Bum + HEPES (Bum/Hep), $n = 4$; Eth + HEPES (Eth/Hep), $n = 5$; Bum + Eth (Bum/Eth), $n = 6$; or all 3 together (Bu/He/Et, $n = 6$).] These responses are displayed as pairs of bars at *right* for each labeled intervention. There is no effect of Time alone. Eth mildly inhibits contraction at 5-min time point. Bum and HEPES significantly suppress contractions to NE. Bum/Hep appears to be more potent than either agent alone. $*P < 0.05$ compared with control response.

presses NE-induced contractions. When both HEPES and bicarbonate are included as buffers ($n = 4$), NE-induced contractions are unaltered. To control for nonspecific effects of bumetanide or HEPES, experiments similar to those in Fig. 6 were carried out using K^+ as the contractile agent (Fig. 8). K^+-induced contractions were obtained that were similar in size to those achieved with NE (~ED_{80}); the mean concentration of K^+ required was 38 mM. Neither bumetanide ($n = 5$) nor HEPES buffer ($n = 5$) had any significant inhibitory effect on K^+-induced contractions.

Cl^--channel blockers inhibit contractile responses to NE (Fig. 9). Four different compounds with the previously documented ability to inhibit anion currents were assayed for their ability to inhibit contractile responses to NE or K^+. The design of these experiments was identical to that of the experiments in Fig. 6. After a control ~ED_{80} response to the agonist and subsequent washout, 10-min incubations in either DIDS (10^{-3} M; $n = 5$ for NE, $n = 6$ for KCl), A-9-C (10^{-3} M; $n = 5$ for NE, $n = 6$ for KCl), niflumic acid (10^{-4} M; $n = 5$ for NE, $n = 4$ for KCl), or tamoxifen (10^{-5} M; $n = 6$ for NE, $n = 5$ for KCl) were performed before reexposure to the agonist. DIDS, A-9-C, and niflumic acid all markedly inhibited NE-induced contractions. Of these three compounds, only DIDS was without effect on KCl-induced contraction. Tamoxifen had no significant effect on contractile responses to either vasoconstrictor.

We wanted to further test the hypothesis that the effect of altering E_{Cl} on NE-induced contraction was

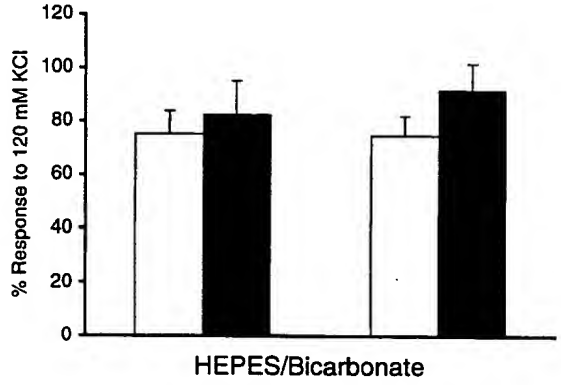


Fig. 7. Effect of HEPES buffer on NE-induced contractions is due to absence of bicarbonate, not to presence of HEPES. When both 10 mM HEPES and the usual 14.9 mM bicarbonate were included in buffer (*right*), control contractile responses to NE (*left*) were not altered. Open bars, 5 min; solid bars = 20 min.

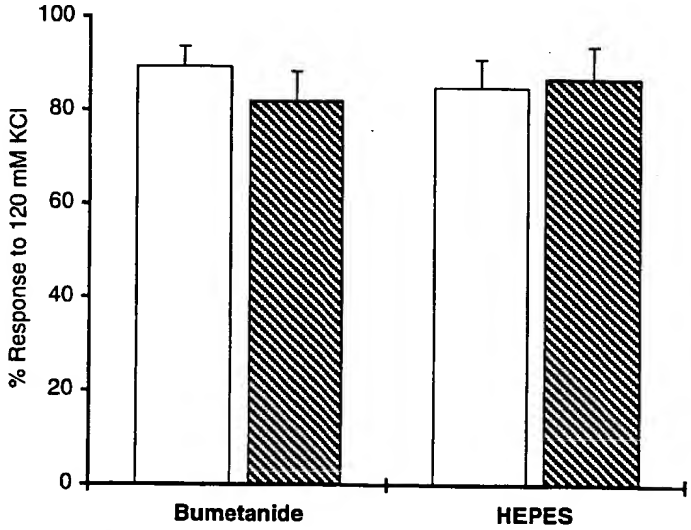


Fig. 8. KCl-induced contractions are not altered by low-Cl^- conditions. Each bar represents tension measured at 20-min time point. Only a single time point was recorded because responses to this dose of KCl never show an initial peak. Open bars represent control contractions, and hatched bars represent tension obtained in response to same dose of KCl after 20 min of exposure to either 10^{-5} M bumetanide ($n = 5$) or 10 mM HEPES buffer ($n = 5$). Neither had any effect on contractions induced by KCl.

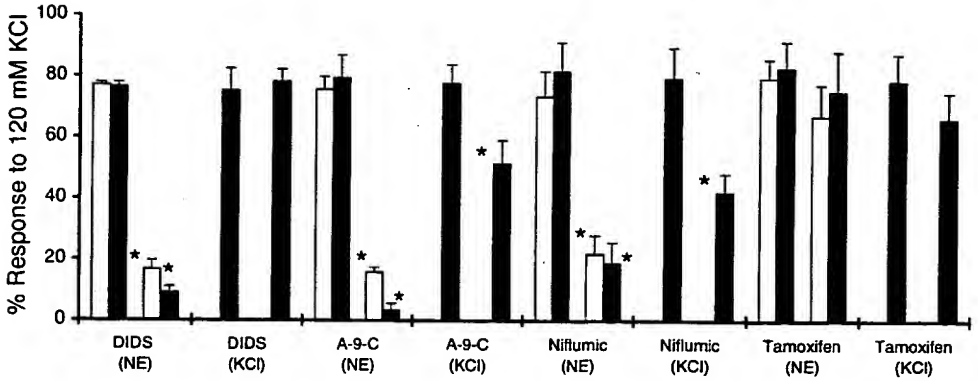


Fig. 9. Cl^--channel blockers inhibit NE-induced contractions. Control responses to NE or KCl were obtained that were ~80% of response to 120 mM KCl (bar or pair of bars at *left* for each labeled intervention). For NE responses, open bars represent highest tension recorded in first 5 min and solid bars represent tension at 20 min. Only tension at 20 min is recorded for KCl-induced contractions. After a 10-min incubation in DIDS (10^{-3} M), anthracene-9-carboxylic acid (A-9-C; 10^{-3} M), niflumic acid (10^{-4} M), or tamoxifen (10^{-5} M), repeat responses were obtained (bar or pair of bars at *right* for each labeled intervention). DIDS, A-9-C, and niflumic acid all inhibited responses to NE, whereas A-9-C and niflumic acid also inhibited KCl-induced contractions. $*P < 0.05$ compared with control response.

dependent on the release of intracellular Ca^{2+}. We therefore used either CPA (10^{-7} M, $n = 5$) to inhibit uptake of Ca^{2+} into the SR or ryanodine (10^{-5} M, $n = 5$) to prevent the release of SR Ca^{2+} (Fig. 10). It was not possible to employ a combination of these two agents because this combination of drugs consistently resulted in large contractions. After an initial control ~ED_{20} response to NE was obtained, rings were continuously exposed to CPA or ryanodine for the remainder of the experiment. Two subsequent control responses to the same dose of NE were elicited to establish a meaningful control response in the presence of the drugs. The effect of ryanodine on the control responses was apparent in that the larger initial peak response to NE was lost and contractions were slower and larger at the 20-min time point. Both CPA and ryanodine diminished the size of control responses to NE. A final response to NE was then obtained in 8 mM Cl^- buffer (MS substituted) containing CPA or ryanodine. This response to NE tended to be larger than the second control response obtained in the presence of the drugs; however, the increase in the peak response to NE seen in low-Cl^- buffer was no longer statistically significant (CPA 2nd control = 13.4 ± 2.4, CPA/low Cl^- = 29.2 ± 9.4, P = 0.11; ryanodine 2nd control = 7.2 ± 2.2, ryanodine/low Cl^- = 19.6 ± 8.1, $P = 0.08$). One can assess the effect of these drugs on the potentiation due to low Cl^- by comparing potentiation as a percentage of the 120 mM KCl response. For example, if the control peak is 21% of the KCl response and the peak in low Cl^- is 57% of the KCl response, then there is an increase of 36%. Under control conditions the peak response to NE is increased in low Cl^- by 35.6 ± 4.7% of the response to 120 mM KCl (Fig. 1, $n = 9$). In CPA, low Cl^- increases the peak response by 7.6 ± 6.4% compared with the initial drug-free control response and by 15.8 ± 8.6% compared with the second response ($n = 5$). Similarly, for ryanodine the increase compared with the drug-free control is 0.8 ± 7.9% and that compared with the second drug-treated control is 12.4 ± 5.9% ($n = 5$). All of these percent increases are statistically smaller in the presence of the drugs (unpaired t-test). In contrast to their suppression of the peak response, these drugs appear to augment the ability of low Cl^- to potentiate the maintained phase of contraction.

DISCUSSION

We have used a variety of approaches to gather data in support of the concept that NE-induced VSM contraction is dependent on activation of a Cl^- current. Cl^- currents appear to contribute to the maintenance of tension not only during the initial phase of the response, when $I_{Cl,Ca}$ has been shown to be activated by the release of SR Ca^{2+}, but also during the maintained phase of contraction. We have shown that NE-induced contraction is potentiated in low-Cl^- buffer (MS substituted) regardless of when extracellular Cl^- is lowered during the contractile response. In contrast, responses to KCl, BAY K 8644, and NE in the absence of extracellular Ca^{2+} are not affected by altering extracellular Cl^-. These other stimuli lack either the ability to release Ca^{2+} stores and thereby activate Cl^- current (KCl, BAY K 8644) or the ability for the depolarization produced by that current to facilitate inward Ca^{2+} flux (NE in 0 Ca^{2+}). The potentiating effect of low Cl^- on NE-induced contraction is concentration and anion dependent. Unlike MS substitution, I^- or Br^- substitution causes suppression of the peak response to NE. These data suggest that I^- and Br^- pass through the channels that are activated by NE more readily than does Cl^-. This implied relative conductivity sequence ($I^- > Br^- > Cl^- >$ MS) is consistent with that recorded for spontaneous inward Cl^- currents characterized in rat portal vein (39). Drugs that interfere with either Cl^- transport (bumetanide, HEPES) or Cl^--channel function (DIDS) inhibit contractile responses to NE but not to KCl. Although the endothelium was left intact in these experiments, the effect of these agents is not due to stimulation of endothelial nitric oxide release (26).

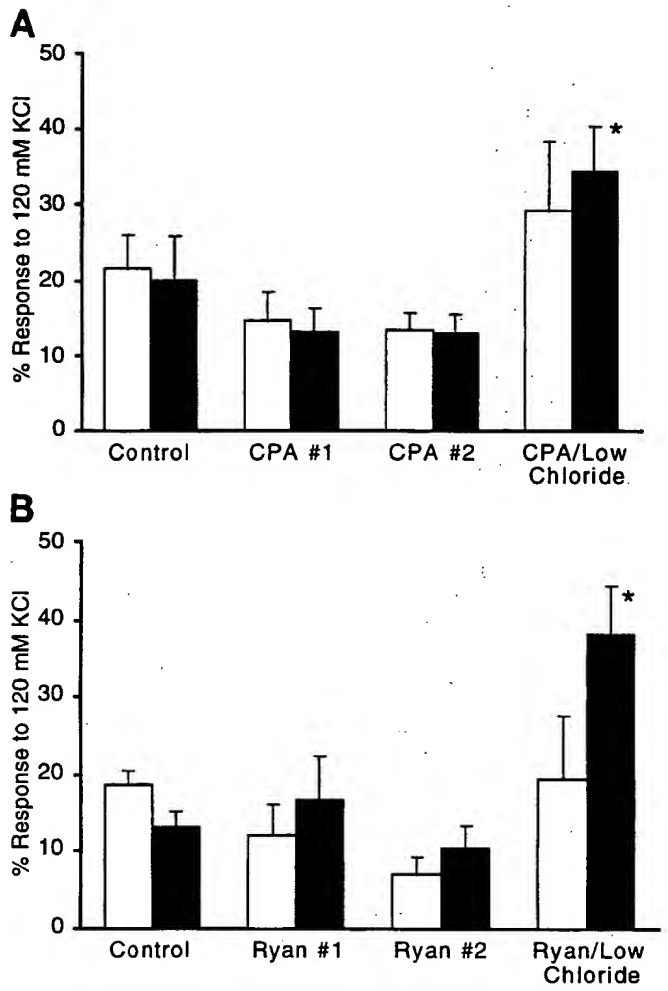


Fig. 10. Effect of cyclopiazonic acid (CPA; 10^{-7} M) (*A*) or ryanodine (Ryan; 10^{-5} M) (*B*) on NE-induced contractions. Data represent peak (open bars) or 3-min (#1) and 20-min (#2) time points (solid bars). First, a control response was obtained that was ~20% of response to 120 mM KCl. Rings were then incubated for 20 min in CPA or ryanodine, and these drugs remained in the bath for the rest of the experiment (including during washout between responses). Two subsequent contractile responses were recorded in presence of drugs to obtain an assessment of the effect of these compounds on NE-induced contractions in normal Cl^- buffer. Subsequent responses were diminished in size, and ryanodine clearly altered time course of contraction by suppressing initial peak response. The final response was obtained in low-Cl^- (8 mM) buffer. *$P < 0.05$ compared with second control response in presence of drugs.

Finally, the Cl^- current responsible for this depolarization is at least partially dependent on agonist-induced release of SR Ca^{2+} stores, because CPA and ryanodine alter the potentiating effect of low-Cl^- buffer. These findings are summarized in Fig. 11.

We propose that the mechanism of the potentiating effect of low-Cl^- buffer is related to the associated change in E_{Cl}. MS is a very impermeable inert anion that has been used previously without discernible biological effects aside from those related to the altered Cl^- concentration (27, 42). We changed Cl^- concentrations abruptly and either simultaneously with or 20 min after exposure to the contractile agent. In both instances, the degree of potentiation decreased with time but remained significant even after 20 min. Previous studies have in fact suggested that intracellular Cl^- begins to fall measurably within 1–2 min following reduction of extracellular Cl^- to 15 mM (15). In evaluating the dose-response to Cl^- (Fig. 4), it is important to note that although the changes in extracellular Cl^- were made on a linear scale, the effect of these changes on E_{Cl} is logarithmic. For this reason, changes in extracellular Cl^- will have a more profound effect on E_{Cl} at lower Cl^- concentrations. For example, with the assumption of an intracellular Cl^- of 44 mM, as has been measured in the rat femoral artery (14), changing extracellular Cl^- from 139 to 106 mM will change E_{Cl} from −30 to −23 mV (7 mV), whereas dropping extracellular Cl^- from 41 to 8 mM changes E_{Cl} from +2 to +44 mV (42 mV). This explains why there is little effect of lowering extracellular Cl^- until relatively low concentrations are reached.

While it is impossible for serum Cl^- ever to fall low enough for alterations in E_{Cl} to effect vascular reactivity in vivo, large changes in E_{Cl} may be achieved by even modest elevations in intracellular Cl^-, and these changes may have physiological significance. Davis et al. (14) have shown that mineralocorticoid-induced (DOCA-salt) hypertension in the rat is associated with a significant rise in intracellular Cl^- in femoral artery VSM (34 vs. 52 mM in bicarbonate-free buffer). This difference can be attributed to an increase in the activity of the Na^+-K^+-2Cl^- cotransporter. If one assumes a serum Cl^- concentration of 100 mM, this change in intracellular Cl^- concentration translates to a shift in E_{Cl} from −29 to −18 mV. This will produce more depolarization when Cl^- conductance dominates V_m, as it may when a contractile agonist binds. This phenomenon may explain at least a portion of the shift in sensitivity to a variety of agonists that is seen in DOCA-salt hypertension.

Producing a depolarizing anion current requires Cl^- to be accumulated intracellularly by transport against its electrochemical gradient. Interfering with this accumulation leaves Cl^- passively distributed, and hence, no depolarizing Cl^- current can flow regardless of how

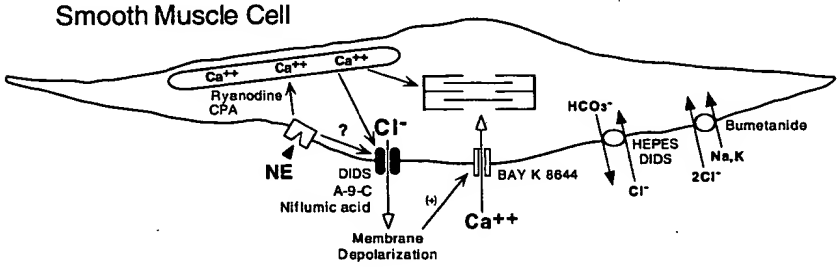


Fig. 11. Model smooth muscle cell outlining proposed mechanism by which Cl^- currents produce vascular smooth muscle depolarization. Important structures are labeled with the pharmacological tool affecting their function. See text for detailed discussion.

many channels are open. VSM accumulates Cl^- via Na^+-K^+-$2Cl^-$ cotransport and Cl^-/HCO_3^- exchange (13). Bumetanide specifically inhibits Na^+-K^+-$2Cl^-$ cotransport (8), whereas the absence of bicarbonate ion interferes with Cl^-/HCO_3^- exchange (13). In guinea pig femoral artery this interference occurs without altering intracellular pH due to a large contribution of Na^+/H^+ exchange to pH regulation (1). Although we have not measured intracellular pH in our tissues, HEPES buffering had no effect on KCl-induced contraction, suggesting that the contractile capability of the rings remained intact. The lack of effect of HEPES on NE-induced contraction in the presence of bicarbonate suggests that, indeed, its effects can be attributed to the absence of bicarbonate. The ability of bumetanide and HEPES to inhibit NE-induced contraction suggests that both of these transporters play a vital role in maintaining the ability of VSM to respond to vasoconstrictors. Ethacrynic acid had only a tiny effect on contractile responses to NE. This may be related to compensatory activity of the other two transport mechanisms or may indicate that this third transporter is not important in aortic tissue.

A number of compounds have been shown to block Cl^- channels; however, none have proven ideal for sorting out the contribution of Cl^- currents to cell activation. DIDS, A-9-C, and niflumic acid all potently inhibit spontaneous transient inward currents produced by $I_{Cl,Ca}$ in rabbit portal vein (19, 20). They do this at lower concentrations (IC_{50}: 2.1×10^{-4} M for DIDS, 3×10^{-4} M for A-9-C, and 2.3×10^{-6} M for niflumic acid at -50 mV) than those at which they inhibit agonist (NE, caffeine)-induced Cl^- current (IC_{50}: 7.5×10^{-4} M for DIDS, 6.5×10^{-4} M for A-9-C, and 6.6×10^{-6} M for niflumic acid). In addition, these compounds have no effect on spontaneous transient outward currents produced by Ca^{2+}-activated K^+ currents ($I_{K,Ca}$), but they clearly augment agonist-induced K^+ currents. These data suggest that these compounds, in addition to blocking Cl^- currents, may also enhance release of Ca^{2+} from the SR (19, 20). We have looked at the ability of each of these agents to inhibit contraction. The concentration of DIDS and A-9-C (10^{-3} M) used to inhibit contraction is only slightly higher than the IC_{50} for inhibition of agonist-induced current. However, we obtained inconsistent inhibition of NE-induced contraction with 10^{-5} M niflumic acid. We therefore carried out our experiments at a concentration of 10^{-4} M. This result is somewhat in contrast to data from Criddle et al. (12) showing that 10^{-5} M niflumic acid produced 38% inhibition of a maximal response to NE (10^{-6} M) and 55% inhibition of rat aortic ring contractions produced by brief exposure (30 s) to this same dose of NE. It seems unlikely that any of these agents interfere with α-adrenergic activation of the tissue because NE-induced $I_{K,Ca}$ is not blocked (19, 20). Of the agents used, DIDS appears to be both highly effective and specific for NE-induced contraction over KCl-induced contraction. The effectiveness of DIDS in these experiments may be enhanced by its ability to block not only Cl^- channels but also Cl^- transport (13). The mechanism by which A-9-C and niflumic acid inhibit KCl-induced contraction is not clear. We have shown previously (27) that neither DIDS nor niflumic acid inhibit voltage-dependent Ca^{2+} current directly. Tamoxifen, which has been shown to inhibit swelling-induced Cl^- currents nearly completely at 10^{-5} M (41), had no effect on the response to NE. On the basis of the lack of effect of this drug in our system, these channels do not appear to play a role in NE-induced VSM activation.

If agonist-induced SR Ca^{2+} release is the primary trigger for activation of Cl^- current, we hypothesized that compounds that interfere with SR function such as ryanodine and CPA should prevent the potentiation produced by low-Cl^- buffer. Indeed, we saw a reduction in the potentiation of the initial peak of contraction. However, the increase in contraction at the 20-min time point was actually more dramatic in the presence of these drugs. The most likely explanation for this is that, in addition to altering the contractile response of the VSM, these compounds may also induce SR dysfunction in the endothelium, resulting in reduced nitric oxide (NO′) production in response to NE. This issue is explored further in our companion paper (26), which defines the important effect of endothelial NO′ on the response to NE in low-Cl^- buffer. Indeed, NO′ appears to have more influence on agonist-induced Cl^- current during the maintained phase of contraction.

Although it is well established that a low-conductance $I_{Cl,Ca}$ is activated by the release of intracellular Ca^{2+} from the SR (23, 32, 37), it remains unclear over what period of time these channels are active. In the rat portal vein, the channels remain open as long as intracellular Ca^{2+} is elevated in the range over which the channels are active (170–600 nM) (31). The lack of effect of low-Cl^- buffer on KCl-induced contraction would suggest that these channels are not activated by Ca^{2+} entering through voltage-gated channels. This is consistent with our previous patch-clamp results in the rabbit coronary artery (27), in which $I_{Cl,Ca}$ was only activated by depolarizing pulses when SR function was intact and Ca^{2+}-induced Ca^{2+} release could occur. In the presence of caffeine, inward Ca^{2+} currents failed to activate a significant amount of Cl^- current (27). This may represent an insufficient local Ca^{2+} level or may reflect a clustering of Cl^- channels in the vicinity of the SR.

In summary, these data demonstrate that Cl^- currents contribute significantly to VSM activation. This represents the first systematic documentation of the critical functional importance of Cl^- current to agonist-induced VSM contraction. The essential features of our findings are summarized in Fig. 11. Inward transport of Cl^- via Na^+-K^+-$2Cl^-$ cotransport (bumetanide sensitive) and Cl^-/HCO_3^- exchange (inhibited in HEPES buffer) produce an E_{Cl} that is more positive than the V_m. Activation of α-receptors by NE causes the release of intracellular stores of SR Ca^{2+}. This Ca^{2+} activates a Cl^- current, producing Cl^- efflux, depolarization, and activation of voltage-dependent Ca^{2+} channels. It is not clear whether these Cl^- channels can be activated by any other mechanism; however, our data suggest that

the channels remain active long after the initial Ca^{2+} transient (Fig. 2). One might speculate that the high local levels of Ca^{2+} achieved by the release of the SR pool may be required to activate the current; however, lower levels may be adequate to maintain channel activity. Alternatively, a Cl^- conductance other than $I_{Cl,Ca}$ may be active during the sustained phase of contraction. These findings have important implications for our understanding of how vasoconstriction is initiated and maintained.

This work was supported through the Children's Health Research Center at the University of Iowa (National Institute of Child Health and Human Development Grant P30-HD-27748) and by a grant from the American Heart Association, Iowa Affiliate.

Address for reprint requests: F. S. Lamb, Dept. of Pediatrics, 5040C RCP, Univ. of Iowa Hospitals, Iowa City, Iowa 52242.

Received 21 October 1997; accepted in final form 23 March 1998.

REFERENCES

1. **Aickin, C. C.** Regulation of intracellular pH in smooth muscle cells of the guinea-pig femoral artery. *J. Physiol. (Lond.)* 479: 331–340, 1994.
2. **Amedee, T., W. Large, and Q. Wang.** Characteristics of chloride currents activated by noradrenaline in rabbit ear artery. *J. Physiol. (Lond.)* 428: 501–516, 1990.
3. **Bolton, T. B., R. J. Lang, and T. Takewaki.** Mechanisms of action of noradrenaline and carbachol on smooth muscle of the guinea-pig anterior mesenteric artery. *J. Physiol. (Lond.)* 351: 549–572, 1984.
4. **Brayden, J. E., and M. T. Nelson.** Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* 256: 532–535, 1992.
5. **Byrne, N. G., and W. A. Large.** The action of noradrenaline on single smooth muscle cells freshly dispersed from the guinea pig pulmonary artery. *Br. J. Pharmacol.* 91: 89–94, 1987.
6. **Byrne, N. G., and W. A. Large.** Membrane ionic mechanisms activated by noradrenaline in cells isolated from rat portal vein. *J. Physiol. (Lond.)* 404: 557–573, 1988.
7. **Casteels, R., K. Kitamura, H. Kuriyama, and H. Suzuki.** The membrane properties of the smooth muscle cells of the rabbit main pulmonary artery. *J. Physiol. (Lond.)* 271: 41–61, 1977.
8. **Chipperfield, A. R.** The (Na^+-K^+-Cl^-) co-transport system. *Clin. Sci. (Colch.)* 71: 465–476, 1986.
9. **Chipperfield, A. R., J. P. Davis, and A. A. Harper.** An acetazolamide-sensitive inward chloride pump in vascular smooth muscle. *Biochem. Biophys. Res. Commun.* 194: 407–412, 1993.
10. **Chipperfield, A. R., J. P. L. Davis, and A. A. Harper.** An estimate of the permeability ratios of Na, K and Cl^- in rat femoral arterial smooth muscle measured in vitro (Abstract). *J. Physiol. (Lond.)* 446: 51P, 1992.
11. **Clapp, L. H., and A. M. Gurney.** ATP-sensitive K^+ channels regulate resting potential of pulmonary arterial smooth muscle cells. *Am. J. Physiol.* 262 (*Heart Circ. Physiol.* 31): H916–H920, 1992.
12. **Criddle, D. N., R. Soares de Moura, I. A. Greenwood, and W. A. Large.** Effect of niflumic acid on noradrenaline-induced contractions of the rat aorta. *Br. J. Pharmacol.* 118: 1065–1071, 1996.
13. **Davis, J. P.** The effects of Na(+)-K(+)-Cl^- co-transport and Cl(−)-HCO_3^- exchange blockade on the membrane potential and intracellular chloride levels of rat arterial smooth muscle, in vitro. *Exp. Physiol.* 77: 857–862, 1992.
14. **Davis, J. P., A. R. Chipperfield, and A. A. Harper.** Accumulation of intracellular chloride by (Na-K-Cl) co-transport in rat arterial smooth muscle is enhanced in deoxycorticosterone acetate (DOCA)/salt hypertension. *J. Mol. Cell. Cardiol.* 25: 233–237, 1993.
15. **Davis, J. P., A. R. Chipperfield, and A. A. Harper.** Comparison of the electrical properties of arterial smooth muscle in normotensive rats and rats with deoxycorticosterone acetate-salt-induced hypertension: possible involvement of (Na(+)-K(+)-Cl^-) co-transport. *Clin. Sci. (Colch.)* 81: 73–78, 1991.
16. **Davis, J. P. L.** Evidence against a contribution by Na-Cl cotransport to chloride accumulation in rat arterial smooth muscle. *J. Physiol. (Lond.)* 491: 61–68, 1996.
17. **Droogmans, G., G. Callewaert, L Declerck, and R. Casteels.** ATP-induced Ca^{2+} release and Cl^- current in cultured smooth muscle cells from pig aorta. *J. Physiol. (Lond.)* 440: 623–634, 1991.
18. **Gelband, C. H., and J. R. Hume.** $[Ca^{2+}]_i$ inhibition of K^+ channels in canine renal artery. Novel mechanism for agonist-induced membrane depolarization. *Circ. Res.* 77: 121–130, 1995.
19. **Hogg, R. C., Q. Wang, and W. A. Large.** Action of niflumic acid on evoked and spontaneous calcium-activated chloride and potassium currents in smooth muscle cells from rabbit portal vein. *Br. J. Pharmacol.* 112: 977–984, 1994.
20. **Hogg, R. C., Q. Wang, and W. A. Large.** Effects of Cl channel blockers on Ca-activated chloride and potassium currents in smooth muscle cells from rabbit portal vein. *Br. J. Pharmacol.* 111: 1333–1341, 1994.
21. **Jones, A. W.** Content and fluxes of electrolytes. In: *Handbook of Physiology. The Cardiovascular System. Vascular Smooth Muscle.* Bethesda, MD: Am. Physiol. Soc., 1980, sect. 2, vol. II, chapt. 11, p. 253–299.
22. **Kajiwara, M., K. Kitamura, and H. Kuriyama.** Neuromuscular transmission and smooth muscle membrane properties in guinea-pig ear artery. *J. Physiol. (Lond.)* 315: 283–302, 1981.
23. **Klockner, U.** Intracellular calcium ions activate a low-conductance chloride channel in smooth-muscle cells isolated from human mesenteric artery. *Pflügers Arch.* 424: 231–237, 1993.
24. **Klockner, U., and G. Isenberg.** Endothelin depolarizes myocytes from porcine coronary and human mesenteric arteries through a calcium-activated chloride current. *Pflügers Arch.* 418: 168–175, 1991.
25. **Kreye, V. A., R. Kern, and I. Schleich.** ^{36}Chloride efflux from noradrenaline-stimulated rabbit aorta inhibited by sodium nitroprusside and nitroglycerine. In: *Excitation-Contraction Coupling in Smooth Muscle*, edited by R. Casteels. Amsterdam, The Netherlands: Elsevier/North-Holland, 1977, p. 145–150.
26. **Lamb, F. S., and T. J. Barna.** The endothelium modulates the contribution of chloride currents to norepinephrine-induced vascular contraction. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H161–H168, 1998.
27. **Lamb, F. S., K. A. Volk, and E. F. Shibata.** Calcium-activated chloride current in rabbit coronary artery myocytes. *Circ. Res.* 75: 742–750, 1994.
28. **Mekata, F., and H. Niu.** Biophysical effects of adrenaline on the smooth muscle of the rabbit common carotid artery. *J. Gen. Physiol.* 59: 92–102, 1972.
29. **Nelson, M. T., J. B. Patlak, J. F. Worley, and N. B. Standen.** Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol.* 259 (*Cell Physiol.* 28): C3–C18, 1990.
30. **Nilius, B., J. Sehrer, F. Viana, C. De Greef, L. Raeymaekers, J. Eggermont, and G. Droogmans.** Volume-activated Cl currents in different mammalian non-excitable cell types. *Pflügers Arch.* 428: 364–371, 1994.
31. **Pacaud, P., G. Loirand, G. Gregoire, C. Mironneau, and J. Mironneau.** Calcium-dependence of the calcium-activated chloride current in smooth muscle cells of rat portal vein. *Pflügers Arch.* 421: 125–130, 1992.
32. **Pacaud, P., G. Loirand, J. L. Lavie, C. Mironneau, and J. Mironneau.** Noradrenaline activates a calcium-activated chloride conductance and increases the voltage-dependent calcium current in cultured single cells of the rat portal vein. *Br. J. Pharmacol.* 97: 139–146, 1989.
33. **Saiguse, A., and S. Kokubun.** Protein kinase C may regulate resting anion conductance in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 155: 882–889, 1988.
34. **Smith, J. M., and A. W. Jones.** Calcium-dependent fluxes of potassium-42 and chloride-36 during norepinephrine activation of rat aorta. *Circ. Res.* 56: 507–516, 1985.

35. **Suzuki, H.** Increase in membrane resistance during noradrenaline-induced depolarization in arterial smooth muscle. *Jpn. J. Physiol.* 36: 433–440, 1986.
36. **Van Helden, D. F.** An alpha-adrenoreceptor-mediated chloride conductance in mesenteric veins of the guinea-pig. *J. Physiol. (Lond.)* 401: 489–501, 1988.
37. **Van Renterghem, C., and M. Lazdunski.** Endothelin and vasopressin activate low conductance chloride channels in aortic smooth muscle cells. *Pflügers Arch.* 425: 156–163, 1993.
38. **Wahlstrom, B. A.** Ionic fluxes in the rat portal vein and the applicability of the Goldman equation in predicting the membrane potential from flux data. *Acta Physiol. Scand.* 89: 436–448, 1973.
39. **Wang, Q., R. C. Hogg, and W. A. Large.** Properties of spontaneous inward currents recorded in smooth muscle cells isolated from the rabbit portal vein. *J. Physiol. (Lond.)* 451: 525–537, 1992.
40. **Yuan, X. J.** Voltage-gated K^+ currents regulate resting membrane potential and $[Ca^{2+}]_i$ in pulmonary arterial myocytes. *Circ. Res.* 77: 370–378, 1995.
41. **Zhang, J. J., T. J. C. Jacob, M. A. Valverde, S. P. Hardy, G. M. Mintenig, F. V. Sepulveda, D. R. Gill, S. C. Hyde, A. E. O. Trezise, and C. F. Higgins.** Tamoxifen blocks chloride channels; a possible mechanism for cataract formation. *J. Clin. Invest.* 94: 1690–1697, 1994.
42. **Zygmunt, A. C., and W. R. Gibbons.** Calcium-activated chloride current in rabbit ventricular myocytes. *Circ. Res.* 68: 424–437, 1991.

The endothelium modulates the contribution of chloride currents to norepinephrine-induced vascular contraction

FRED S. LAMB AND THOMAS J. BARNA
Department of Pediatrics, University of Iowa, Iowa City, Iowa, 52242

Lamb, Fred S., and Thomas J. Barna. The endothelium modulates the contribution of chloride currents to norepinephrine-induced vascular contraction. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H161–H168, 1998.—Activation of a Cl^- current is critical to agonist-induced activation of rat aortic smooth muscle contraction. Substituting extracellular Cl^- with 130 mM methanesulfonate (8 mM Cl^-) increases the contractile response to norepinephrine (NE) but not to KCl. We hypothesized that endothelial factors modulate this effect. Removing the endothelium (rubbing) or treatment with *N*-nitro L-arginine (L-NNA) markedly increased the potentiation of NE-induced contraction by low-Cl^- buffer. Indomethacin had no effect. The previously demonstrated ability of Cl^--channel blockers (DIDS, anthracene-9-carboxylic acid, niflumic acid) or Cl^- transport inhibitors (bumetanide, bicarbonate-free buffer) to inhibit responses to NE was not altered by L-NNA. Low-Cl^- buffer alone did not contract intact rings but produced nifedipine-sensitive contractile responses after rubbing or L-NNA treatment. These data suggest that the Cl^- conductance of smooth muscle in intact blood vessels is low but increases with withdrawal of reduced nitric oxide (NO′) or agonist stimulation. Rubbing or L-NNA increased the sensitivity of rings to KCl but not to NE. Nifedipine reduced both sensitivity and maximum response to NE in intact vessels. L-NNA increased the maximum response to NE in nifedipine-treated rings without changing sensitivity. We conclude that although NO′ affects both the voltage-dependent and voltage-independent components of contraction, sensitivity to NE is determined by the voltage-dependent portion. The voltage change required for a full response to NE is dependent on activation of a Cl^- current that may be under the tonic regulatory influence of NO′.

nitric oxide; *N*-nitro-L-arginine; nifedipine; indomethacin; methanesulfonate

THE ENDOTHELIUM INFLUENCES the contractile function of vascular smooth muscle (VSM) via the release of a variety of vasoconstrictor and vasodilator agents [prostaglandins, thromboxanes, reduced nitric oxide (NO′), endothelium-derived hyperpolarizing and contracting factors (EDHF, EDCF)]. Changes in the basal production of NO′, in particular, can alter smooth muscle responsiveness to vasoconstrictors (7, 15, 20). In addition, increasing quantities of NO′ may be produced in response to vasoconstrictor agents, thus providing a brake on the process of contraction (7a). We have some insight into the cellular mechanisms by which NO′ acts on membrane ion channels to produce smooth muscle relaxation. There is considerable evidence that NO′ can activate K^+ channels, resulting in membrane hyperpolarization, inhibition of voltage-dependent Ca^{2+} current, and smooth muscle relaxation (18). NO′ may also inhibit Ca^{2+}-channel activity via cGMP (5, 10, 14, 16). We propose that these mechanisms by which NO′ produces relaxation may be supplemented by an ability to interfere with agonist-induced activation of Cl^- current, thus interfering with depolarization and preventing contraction.

We have presented evidence that agonist-induced contraction of vascular smooth muscle depends on the activation of a Cl^- current that has the permeability sequence $I^- > Br^- > Cl^-$ > methanesulfonate (MS) (8). This Cl^- current may be activated by the release of stored intracellular Ca^{2+}. It is proposed that the ensuing depolarization is essential for producing contraction-sustaining entry of Ca^{2+} through voltage-dependent Ca^{2+} channels. Inhibiting or enhancing the activation of depolarizing Cl^- current may be as important to regulation of VSM membrane potential as is the activation or inhibition of K^+ current.

Because Cl^- conductance appears to be quite low at rest (3) and is activated to a large degree by agonists (2, 6, 17), it may be important to distinguish between those mechanisms that cause the relaxation of an established contraction and those that may prevent vasoconstriction. These may represent two quite distinct cellular processes. If Cl^- current is responsible for a portion of sustained agonist-induced depolarization, then, similar to the activation of K^+ current, inhibition of Cl^- current may relax an established contractile response. Neither K^+ channel activation nor Cl^- channel inhibition will have any effect, however, if the cell is not first depolarized by the agonist and contraction initiated. Interfering with this process may provide a potent mechanism for regulating vascular contractility. Understanding this distinction between vasodilatation and suppressed contractility will require an in-depth understanding of the cellular processes involved in VSM contraction, including the mechanisms regulating depolarization in response to an agonist.

The current studies were designed to determine whether the agonist-induced Cl^- current of rat aortic VSM is regulated by two endothelial-derived factors that inhibit contraction, cyclooxygenase products of arachadonate metabolism (prostacyclin) or NO′. We have also addressed the issue of how NO′ controls the sensitivity of the VSM response to adrenergic stimulation. The results suggest that NO′ may regulate both resting Cl^- conductance and the ability of agonists to activate the Cl^- current required for depolarization and contraction. Furthermore, it appears that NO′ regulates vascular sensitivity to catecholamines by altering this voltage-dependent (nifedipine-sensitive) portion of the contractile response. This may occur by a combination of several mechanisms including *1*) controlling the response of Ca^{2+} channels to a given degree of depolarization, *2*) preventing depolarization via inhibition of Cl^- channels, or *3*) limiting depolarization via activation of K^+ channels.

METHODS

Rings of thoracic aorta were obtained from adult male Sprague-Dawley rats and prepared for isometric force recording in a manner identical to that described in our preceding companion article (8). Control and low-Cl^- buffers were also prepared in the same manner. Low-Cl^- buffer in this study refers to 8 mM Cl^- substituted with 130 mM MS. The endothelium was removed from some rings by gently rolling the tissue around the end of a finely serrated steel forceps. Indomethacin treatment consisted of a 1-h exposure at 10^{-6} M, which occurred during the 2-h equilibration period that preceded all experiments. NO synthesis was inhibited by incubation in *N*-nitro-L-arginine (L-NNA; 10^{-4} M) for 20 min before a response was elicited. Dose-response experiments were performed cumulatively, and the response to each concentration of norepinephrine (NE) was observed for 10 min before the next higher concentration was added. This time period was generally adequate to allow a plateau of the contractile response. All drugs and all salts for the preparation of physiological salt solution were obtained from Sigma Chemical.

Data are displayed as means ± SE. Calculations of the half-maximal effective dose (ED_{50}) were performed by linear regression of each dose-response curve following logit transformation of the response data and log transformation of the agonist concentrations. The resulting linear equations were then solved for the dose producing the half-maximal response. Statistical analysis of group differences was performed using Student's *t*-test, and *n* values represent the number of animals in each group. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

There are two readily recognized phases to VSM contraction. The initial contractile response has been attributed to activation of the contractile proteins by Ca^{2+} released from the sarcoplasmic reticulum. Our hypothesis maintains that a second important function of this Ca^{2+} pool is to activate a depolarizing Cl^- current. This depolarization results in the influx of extracellular Ca^{2+}, which contributes to the second, or maintained phase, of contraction. To assess the effect of endothelial factors on both phases of contraction, tension was recorded at both an early and a late time point. As previously demonstrated (8), the response of intact aortic rings to an approximately 20% effective dose ($\sim ED_{20}$) of NE is potentiated in low-Cl^- buffer (Fig. 1, *A* and *B*). The effect is most impressive at the 3-min, or peak, time point but is also statistically significant during the maintained phase of contraction (20 min). This effect of low-Cl^- buffer is not altered by inhibiting prostaglandin production with indomethacin (10^{-6} M). Even in normal buffer, rubbed or L-NNA-treated rings contract more vigorously to a single dose of NE than do intact rings. A contraction to ~20% of the response to 120 mM KCl (ED_{20}) was achieved at a significantly lower concentration of NE in tissues with an impaired ability to synthesize NO' [intact rings, $3.0 \pm 0.5 \times 10^{-8}$ M NE: 19.5 ± 2.7 at 3 min, 15.2 ± 2% at 20 min ($n = 9$); indomethacin-treated rings, $8.3 \pm 2.6 \times 10^{-9}$ M NE: 21.2 ± 4.3% at 3 min, 12.4 ± 3.0% at 20 min ($n = 6$); rubbed rings, $1.2 \pm 0.3 \times 10^{-9}$ M NE: 16.0 ± 2.0% at 3 min, 23.4 ± 3.3% at 20 min ($n = 5$); and L-NNA-treated rings, $1.6 \pm 0.4 \times 10^{-9}$ M NE: 27 ± 7.1% at 3 min, 23.6 ± 4.4% at 20 min ($n = 5$)]. There was no significant difference among these contractile responses at the 3-min, or peak, time point. After 20 min, the response

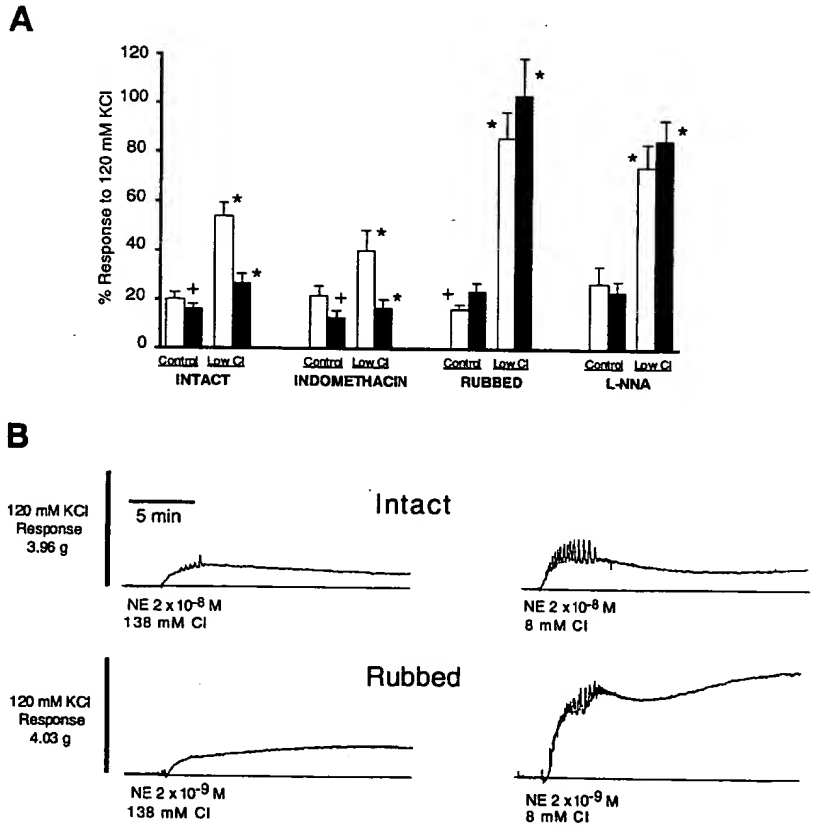


Fig. 1. *A*: low-Cl^- buffer potentiates contractile response to norepinephrine (NE). Tension recorded at both 3 min (peak, open bars) and 20 min (steady state, solid bars) is significantly increased in intact rings. This effect is not altered by pretreatment with indomethacin. Removal of endothelium by rubbing or inhibition of reduced nitric oxide (NO') synthase with *N*-nitro-L-arginine (L-NNA) results in much greater potentiation of NE response by low Cl^-. $*P < 0.05$, tension in low-Cl^- buffer vs. tension in control; $+P < 0.05$, tension at 3 min vs. tension at 20 min in control rings. *B*: typical tension recordings of response of intact and rubbed rings to NE in control buffer (138 mM Cl^-) and in presence of 8 mM Cl^-. Solid vertical bars (*left*) represent the size of response of that ring to 120 mM KCl. The most remarkable effect of disrupting NO' production is the large increase in steady-state tension recorded at 20 min.

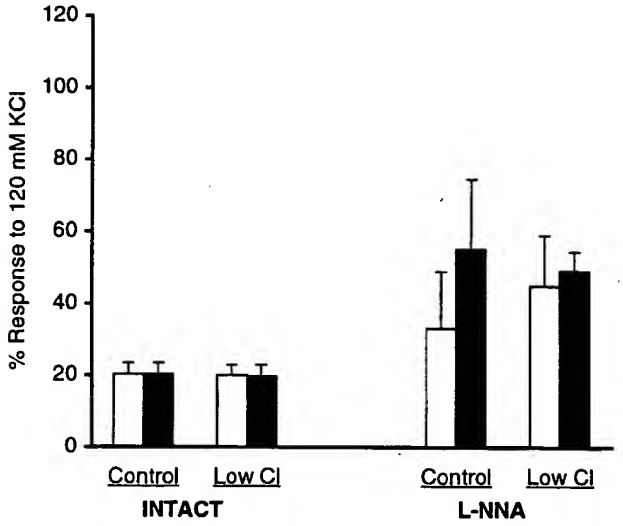


Fig. 2. Low-Cl^- buffer does not alter contractile responses to K^+ at either the 3-min (open bars) or 20-min (solid bars) time point. In intact rings, 18 mM K^+ produces ~20% of maximum response to KCl regardless of Cl^- concentration of buffer. In rings treated with L-NNA, contractile response to K^+ is potentiated; therefore 8 mM K^+ was used. There was no significant effect of low-Cl^- buffer on K^+-induced contraction even when NO synthase was inhibited.

was significantly larger in rubbed or L-NNA-treated rings. This difference was due to a significant drop-off in tension from the peak to the maintained phase of contraction in the intact and indomethacin-treated rings. This drop-off may be attributed to NE-induced NO′ production activated either directly, via endothelial receptors, or indirectly, by stretch. Rubbed and L-NNA-treated rings have a markedly potentiated response to NE in low-Cl^- buffer at both 3 and 20 min. This effect is most prominent on the maintained phase of contraction. The tension at the 20-min time point exceeds that at 3 min. This is in marked contrast to intact and indomethacin-treated rings, which show an even more remarkable time-dependent drop-off in tension in low-Cl^- buffer than in normal buffer. Figure 1*B* shows typical responses of isolated aortic rings with and without endothelium in normal and low-Cl^- buffer.

As previously reported (8), low-Cl^- buffer does not alter contractile responses to K^+ in intact rings (Fig. 2). In these vessels, 18 mM K^+ produced ~20% of the maximum response to KCl regardless of the Cl^- concentration of the buffer. In rings treated with L-NNA, the contractile response to K^+ is potentiated. This potentiation increases with the duration of exposure to L-NNA (data not shown). In L-NNA-treated rings, 8 mM K^+ (KCl in normal buffer and K-MS in low-Cl^- buffer) was used as the agonist because responses to lower concentrations were inconsistent. To control for the time-dependent effect of L-NNA, two rings were prepared from each animal (8 rings from 4 animals). Responses were obtained from both rings in both normal and low-Cl^- buffer, with one ring being exposed to 8 mM K^+ in normal buffer first and the other ring exposed to low-Cl^- buffer first. The responses of the two rings from each animal were averaged, and therefore all eight rings were included in the statistical calculations. In contrast to the effect seen with NE, there was no effect of low-Cl^- buffer on K^+-induced contraction when NO synthase was inhibited.

Because of the dramatic ability of NO′ to alter the potentiation of NE-induced contraction via low Cl^-, we wished to demonstrate that the previously documented effects of Cl^--channel blockers [DIDS (10^{-3} M), anthracene-9-carboxylic acid (10^{-3} M), and niflumic acid (10^{-4} M)] and Cl^- transport inhibitors [bumetanide (10^{-5} M) and bicarbonate-free buffer (10 mM HEPES)] on NE-induced contraction (8) were not related to activation of NO′ release by the endothelium. We therefore repeated these experiments in L-NNA-treated rings (Fig. 3). Inhibition of NO′ production did not impair the ability of these interventions to suppress contractile responses to NE. These compounds appear to exert their effect independent of NO.

Low-Cl^- buffer alone produced little or no contractile response in intact rings (n = 11), and no response was

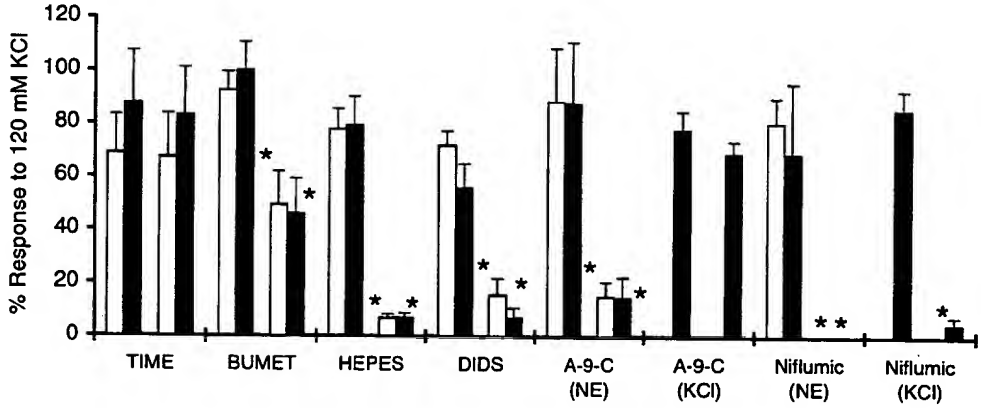


Fig. 3. Ability of Cl^--channel blockers and Cl^- transport inhibitors to interfere with NE-induced contraction is not altered by L-NNA and therefore is not dependent on endothelial release of NO′. Control responses to NE or KCl were obtained that were ~80% of response to 120 mM KCl [pair of bars (NE) or bar (KCl) at *left* for each labeled intervention]. Open bars represent highest tension achieved in the first 5 min; solid bars represent tension at 20 min. Repeat responses (bar or pair of bars at *right* for each labeled intervention) were obtained following a 20-min incubation in normal buffer only (Time control), bumetanide (10^{-5}), or HEPES (Bumet; 10 mM) or a 10-min incubation in DIDS (10^{-3} M), anthracene-9-carboxylic acid (A-9-C; 10^{-3} M), or niflumic acid (10^{-4} M). *$P < 0.05$ vs. control.

ever noted at the 3-min time point, whereas 3 of 11 rings showed very small contractions (2–9% of the response to 120 mM KCl) at the 20-min time point (Fig. 4*A*). In contrast, rubbed rings (n = 21) contracted consistently to low-Cl^- buffer, but to a variable degree. Examples of the spectrum of response to low Cl^- in rubbed rings are shown in Fig. 4*B*. These responses to low-Cl^- buffer were not inhibited by phentolamine (10^{-5} M, n = 3, data not shown). It is possible that the three intact rings that did have small responses to low Cl^- underwent inadvertent partial endothelial damage during isolation.

Intact aortic rings underwent very small contractions in response to L-NNA (10^{-4} M) that developed slowly [0% (3 min) and 3.1 ± 1.2% (20 min) of response to 120 mM KCl]. After a 10-min exposure to L-NNA, these rings contracted to low-Cl^- buffer to a degree similar to that of rubbed rings (Fig. 5*A*). These contractions were also quite variable from ring to ring, suggesting that the variability seen in the response to low Cl^- in rubbed rings was not a function of incomplete denuding but rather was characteristic of the contractile response to low Cl^-. Both the response to L-NNA and the low Cl^--induced contraction are completely inhibited by nifedipine (10^{-6} M, n = 6), suggesting that the low Cl^- response is produced by membrane depolarization that either did not occur or did not result

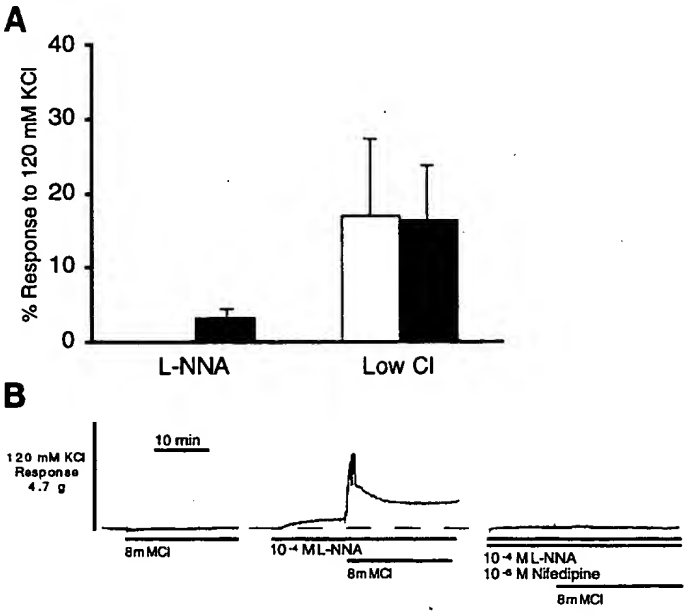


Fig. 5. *A*: intact aortic rings contract in response to L-NNA (10^{-4} M), although these contractions are very small. After a 10-min exposure to L-NNA, rings contract to low-Cl^- buffer to a degree similar to that of rubbed rings (see Fig. 3). Open bars, 3-min time point; solid bars, 20-min time point. *B*: typical response of an intact aortic ring to L-NNA followed by low-Cl^- buffer. Solid vertical bar (*left*) represents the size of the response of that ring to 120 mM KCl. Low-Cl^--induced contractions were completely inhibited by nifedipine (10^{-6} M).

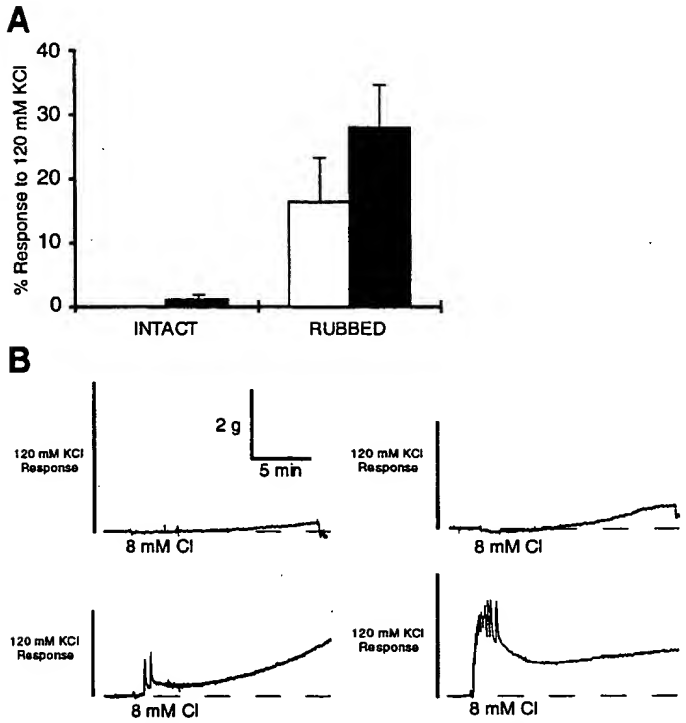


Fig. 4. *A*: low-Cl^- buffer alone produces little or no contractile response in intact rings. No response was ever noted at the 3-min time point (open bars). In rubbed rings a contractile response of inconsistent size is invariably seen. Solid bars, 20-min time point. *B*: typical contractile responses of rubbed rings to low-Cl^- buffer. Solid vertical bars at *left* for each response represent the size of the response of that ring to 120 mM KCl. Note variability of both maximal tension generated and time course of response.

in contraction in the presence of intact endothelial NO′ production (Fig. 5*B*).

Figure 6 shows cumulative dose-response curves to NE in intact rings before and after treatment with L-NNA (n = 7) and in rubbed rings (n = 6). Data in Fig. 6*A* is plotted as tension (g), whereas Fig. 6*B* depicts the data normalized as a percentage of the maximal response of each ring to NE. The rings achieved a greater maximal response to NE than to KCl. The maximal response to NE in intact rings was 7.8 ± 0.29 g, and after treatment with L-NNA this significantly increased to 8.35 ± 0.32 g ($P < 0.05$). The response to 120 mM KCl of these same rings was 4.45 ± 0.20 g. There was no significant difference among the $-\log ED_{50}$ for intact (7.86 ± 0.07), L-NNA-treated (8.00 ± 0.07), or rubbed rings (7.93 ± 0.11). In intact rings, there was a smaller response to NE when it was added as a single dose to produce an $\sim ED_{20}$ response in the low Cl^- experiments ($3.0 \pm 0.5 \times 10^{-8}$ M) than to the same concentration of NE when achieved in a cumulative fashion during a dose-response experiment. The reason for this is not readily apparent, but the difference was not evident in rubbed or L-NNA-treated rings and therefore may be related to how these methods of NE exposure affect endothelial NO′ release.

Cumulative dose-response curves to KCl (Fig. 7) were performed in intact rings before and after exposure to L-NNA (n = 5) and in rubbed rings (n = 4). Rubbing or treatment with L-NNA (10^{-4} M) increased the sensitivity (Fig. 7*B*) of the rings to K^+-induced depolarization (ED_{50}: 17.2 ± 1.2 mM in intact rings;

12.6 ± 0.9 mM in L-NNA-treated rings; 13.9 ± 2.5 mM in rubbed rings). L-NNA treatment also increased the maximum response (Fig. 7*A*) to K^+ in paired experiments (4.57 ± 0.35 g in intact rings, 5.42 ± 0.40 g in L-NAA-treated rings). As previously stated, we find that there is a time-dependent increase in the response to K^+ following rubbing or application of L-NNA. These experiments were performed 20 min after the first and only exposure of the rings to L-NNA.

Nifedipine (10^{-6} M) was used to separate the voltage-dependent portion from the voltage-independent portion of the contractile response to NE. In the presence of nifedipine, both the maximal response (control 7.96 ± 0.29 g, nifedipine 2.28 ± 0.29 g, $n = 5$) (Fig. 8*A*) and the sensitivity (−log ED_{50}: control 7.86 ± 0.7, nifedipine 7.37 ± 0.12) (Fig. 8*B*) to NE are markedly diminished. Treatment of these same rings with nifedipine plus L-NNA caused a large increase in their maximum ability to generate force (4.23 ± 0.51 g); however, there was no recovery of the diminished sensitivity (−log ED_{50}: nifedipine + L-NNA 7.47 ± 0.03, not significantly different from nifedipine alone).

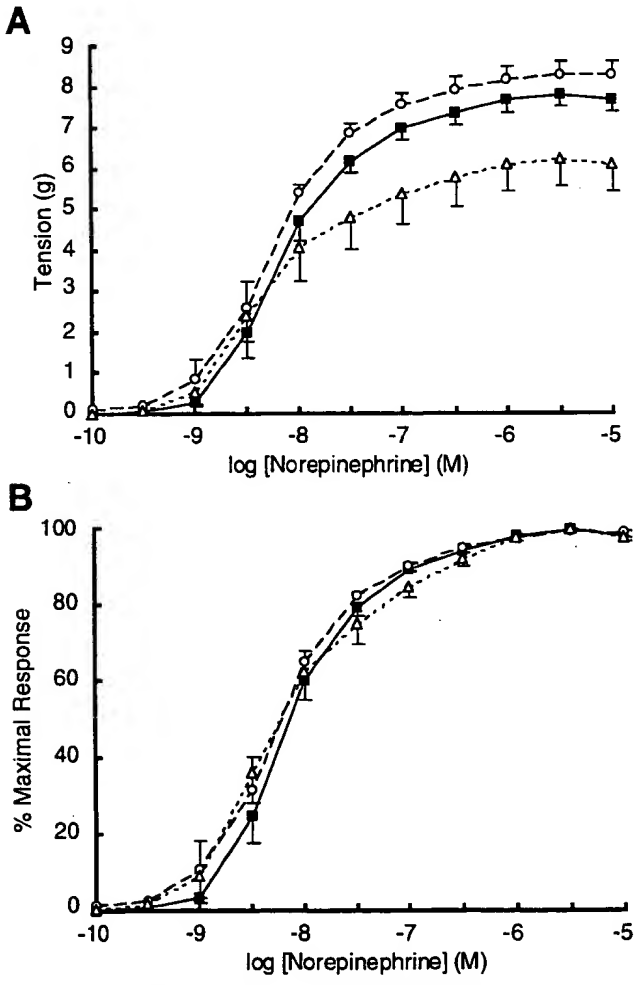


Fig. 6. Concentration-response curves to NE in rat aortic rings. *A*: control rings (■) had an intact endothelium. These responses are compared with those from rubbed (△) and L-NNA-treated rings (○). Rubbing appeared to slightly decrease maximal tension response. *B*: responses were normalized as percentages of maximal tension generation to NE. There was no significant change in sensitivity in rubbed or L-NNA-treated rings.

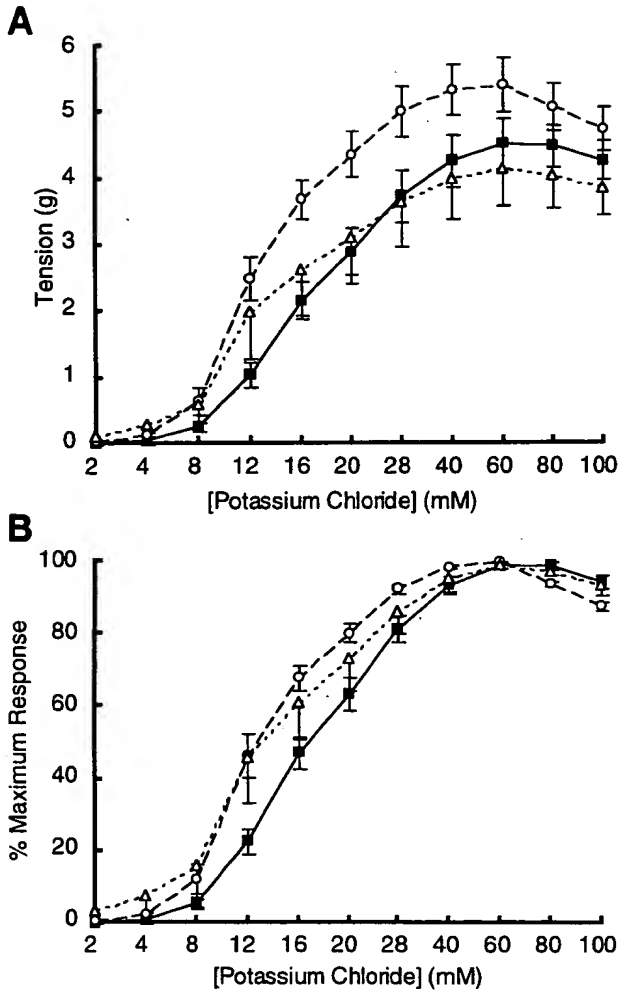


Fig. 7. Concentration-response curves to KCl. *A*: control rings (■) had an intact endothelium. These responses are compared with those from rubbed (△) and L-NNA-treated rings (○). *B*: response are normalized as percentages of maximal tension generation to KCl. Both rubbed and L-NNA-treated rings were significantly more sensitive to K^+-induced depolarization than was control. This suggests that sensitivity to depolarization is under influence of NO′ and is important for determining agonist sensitivity.

DISCUSSION

We have previously shown (8) that NE-induced contraction of the rat aorta is potentiated by low-Cl^- buffer. We now demonstrate that the magnitude of this potentiation is dependent on endothelial NO. Rubbing or L-NNA increased the size of the response to a single low (ED_{20}) concentration of NE. After the dose of NE was reduced to account for this effect, repeating the response in 8 mM Cl^- buffer remarkably enhanced the magnitude of contraction. This effect was particularly pronounced on the maintained (20 min) phase of contraction. Contraction at this time point was mildly potentiated by low-Cl^- buffer when the endothelium was intact (8) but was increased severalfold following

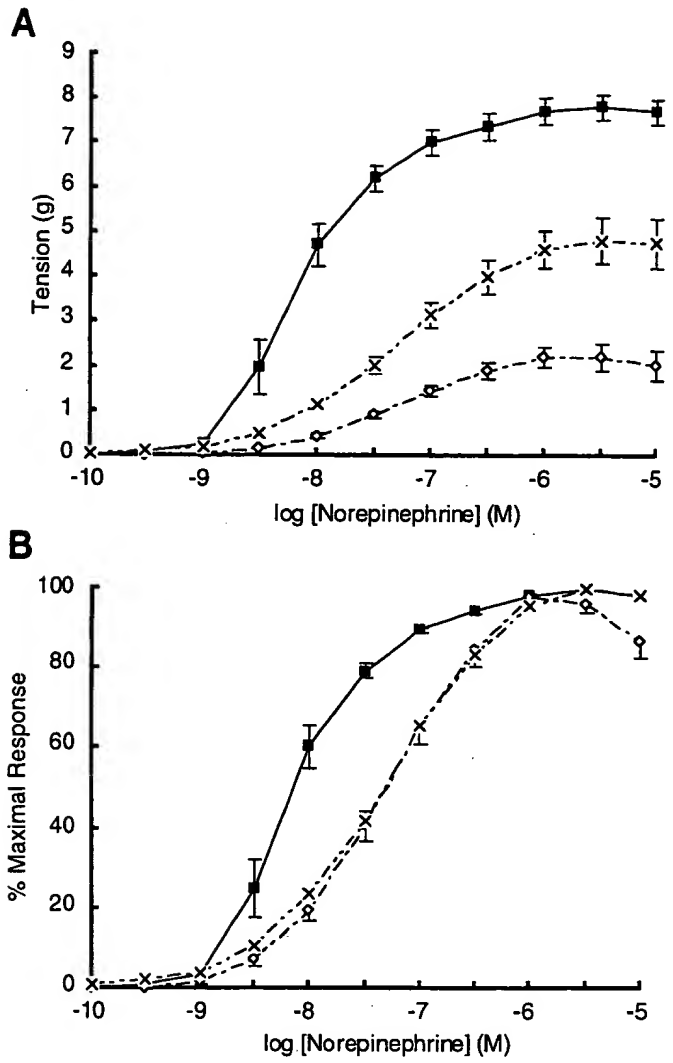


Fig. 8. Concentration-response curves to NE in nifedipine-treated (10^{-6} M) rings with and without L-NNA (10^{-4} M). *A*: control rings (■) had an intact endothelium. These responses are compared with those from rings treated with nifedipine alone (◇) or nifedipine plus L-NNA (×). Non-voltage-dependent component of NE-induced contraction is quite small. *B*: responses are normalized as percentages of maximal tension generated to NE. Sensitivity to the agonist is remarkably diminished by nifedipine. Although inhibition of NO′ synthase does increase the magnitude of voltage-independent response, sensitivity is unaltered by L-NNA. These data suggest that the magnitude of the voltage-independent portion of response to NE is unlikely to be a major determinant of agonist sensitivity.

disruption of endothelial NO′ production. The effects of rubbing and L-NNA were similar, although the control responses in the rubbed rings were slightly smaller and the potentiation by low Cl^- somewhat greater. Any difference between the effects of rubbing and L-NNA would suggest that other endothelial factors such as EDHF may also impact on the response to low Cl^-. Recent data indicate that EDHF acts through activation of K^+ channels (18) and therefore may not directly affect the response to low Cl^-. Prostaglandins do not seem to influence the ability of NE to activate Cl^- current, because pretreatment with indomethacin does not alter the effect of low-Cl^- buffer. K^+-induced contraction is not altered by low Cl^- conditions because elevated extracellular K^+ does not activate a Cl^- conductance.

Low-Cl^- buffer alone does not elicit contraction of intact rings but consistently causes a variable degree of contraction in rubbed or L-NNA-treated rings. This suggests that the resting Cl^- conductance of intact vessels is low enough that even a dramatic change in the Cl^- equilibrium potential does not produce enough depolarization to cause contraction. In the absence of endothelial NO′, the same change in Cl^- gradient elicits a contractile response that is completely blocked by nifedipine and therefore is due to depolarization. These findings suggest that either more Cl^- channels were open at the time of the Cl^- gradient change or more Ca^+ current was activated by the same degree of depolarization. There is ample support from the literature for the regulation of Ca^{2+} channel conductance by cGMP (5, 10, 14, 16). Our results raise the possibility that, in addition to this effect on depolarization-induced Ca^{2+} entry, NO′ may also suppress the degree of depolarization induced by an agonist by inhibiting the activation of Cl^- channel conductance.

We can only indirectly address the question of direct regulation of Cl^- channel conductance by NO′ on the basis of our studies, which assume that contractile responses are proportional to depolarization. If NO′ has a direct interaction with Cl^- channels, then one might expect rubbing or L-NNA to increase the contractile response to NE/low Cl^- more than the response to K^+. These two stimuli represent alternative methods of producing membrane depolarization, and if the effect of NO′ is limited to the Ca^{2+} channel, then they should be similarly affected by NO′ withdrawal. Data from the dose-response to KCl shows that 12 mM K^+ produced an $\sim ED_{20}$ response in intact rings (22.5 ± 3.5%). After exposure to L-NNA, this response is approximately twice as large (46.3 ± 6.2%). In the NE/low-Cl^- studies, in the presence of L-NNA, we used a NE concentration of $1.6 \pm 0.4 \times 10^{-9}$ M to achieve a peak response of 27 ± 7.1% and a maintained response of 23.6 ± 4.4%. This dose of NE is barely a threshold concentration in an intact ring (10^{-9} M NE produced 3.6 ± 1.2% of response to 120 mM KCl, Fig. 6), and a higher dose was required to achieve an $\sim ED_{20}$ response in intact rings ($3.0 \pm 0.5 \times 10^{-8}$ M). Repetition of the response to NE in L-NNA plus low Cl^- (Fig. 1) produced a peak response of 74.4 ± 9.2% and a maintained response of 85.2 ± 8.3%. The maintained response is approximately triple that seen in intact rings. This is even more impressive in view of the higher average concentration of NE used in the intact rings. Unfortunately, this comparison is not completely fair. The response to NE is not simply a function of depolarization. Many additional factors contribute to the magnitude of this response, and we cannot completely separate out the voltage-dependent component. Direct measurement of changes in membrane potential will be required to completely answer this question. The possible ways in which NO′ may be acting to influence our results are summarized in Fig. 9.

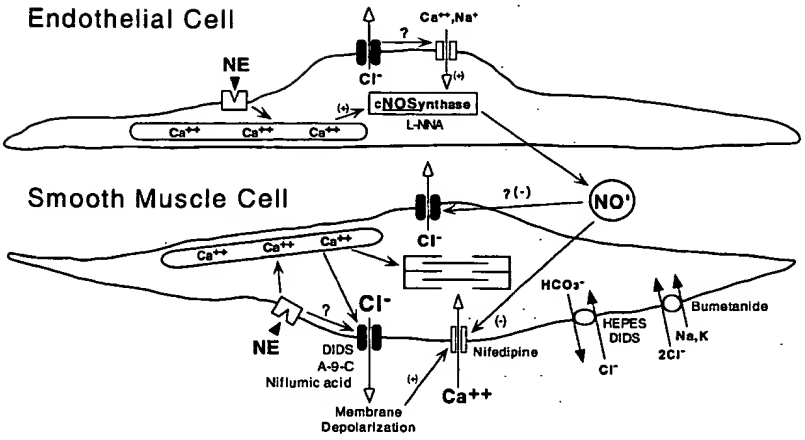


Fig. 9. Model cells outlining possible mechanisms by which endothelial NO may alter Ca^{2+} entry into vascular smooth muscle (VSM). We have performed companion studies (8) delineating the contribution of Cl^- currents to agonist-induced contraction of VSM. Removal of endothelium by rubbing or L-NNA treatment allows a much greater response to low-Cl^- buffer. Several possible explanations may exist: *1*) low-Cl^- buffer increases NO′ release from endothelial cells, thereby suppressing contraction of intact rings; *2*) NO′ suppresses L-type Ca^{2+} channel function, thereby minimizing contractile response to increased depolarization; or *3*) NO′ tonically suppresses Cl^- conductance so that, after withdrawal of NO′, there are more open Cl^- channels and more depolarization is produced when extracellular Cl^- is lowered. In presence of an agonist, NO′ may also alter the degree to which Cl^- conductance can be activated. cNOS, constitutive NO synthase.

One additional factor that could have an impact on the interpretation of our results and that must be considered is the possibility that low-Cl^- buffer can alter endothelial NO′ production directly. If low-Cl^- buffer increased basal NO′ production or augmented an NE-/stretch-induced increase in NO′ production, this could explain the comparatively diminished ability of low Cl^- to augment NE-induced contraction in intact rings. In fact, the literature suggests that just the opposite is true. Endothelial NO′ production appears to be directly proportional to the sustained level of intracellular Ca^{2+} achieved following stimulation by a vasoactive agent (12). This Ca^{2+} enters the cell from extracellular sources through a nifedipine-insensitive, voltage-independent channel (1). Nevertheless, Ca^{2+} influx is apparently controlled by membrane potential in that endothelial cell hyperpolarization increases the driving force for Ca^{2+} influx and thereby increases the sustained level of intracellular Ca^{2+} (11). Conversely, depolarization by high K^+ depresses the agonist-induced sustained increase in intracellular Ca^{2+} (9, 11, 21). Endothelial cells clearly possess Cl^- channels (4, 13, 19), and reduction of extracellular Cl^- (to 20 mM) was found to markedly depress the ATP-induced increase in sustained level of Ca^{2+} in human aortic endothelial cells (21). On the basis of these results, one would predict that low-Cl^- buffer would inhibit rather than augment the sustained release of NO′. This would clearly not explain our results.

The dose-response curve to K^+ is more affected by disruption of NO′ production than is the dose-response curve to NE. The change in the maximal response to NE with L-NNA was significant but quite small. We have no clear explanation for the difference between the response to a single low concentration of NE and the response to cumulatively added NE. Whatever the reason for the difference, it may be a function of NO′ production, because the difference is not seen in rubbed or L-NNA-treated rings. The more pronounced effect of NO′ on KCl-induced contractions may reflect the fact that although this response is completely coupled to depolarization, only a portion of the response to NE is voltage-dependent. We were able to accentuate this portion of the response by lowering extracellular Cl^- and thereby observed a large change in the response to a relatively low concentration of NE.

We have made an attempt to define the voltage-dependent portion of the dose response to NE and to determine how that portion of the response to NE contributes to the sensitivity and magnitude of contraction. Complete inhibition of depolarization-induced Ca^{2+} influx through voltage-dependent channels (10^{-6} M nifedipine) dramatically reduces both the magnitude of contraction and the sensitivity to NE in intact rings (Fig. 8). Addition of L-NNA approximately doubles the size of the contractile response to NE in the presence of nifedipine but has absolutely no effect on sensitivity. This result suggests that NO′ has significant effects on vascular contraction that are independent of Ca^{2+} influx, but these intracellular events do not determine sensitivity. The remarkable L-NNA-induced change in sensitivity to K^+ suggests that Ca^{2+} channels are an important target for regulation of vascular sensitivity by NO′; however, changes in intracellular Ca^{2+} handling may also contribute to this. The large rightward shift in the sensitivity to NE induced by nifedipine suggests that agonist-induced depolarization makes an important contribution to the contractile response even at low agonist concentrations of NE. If this depolarization is indeed Cl^- dependent, Cl^- homeostasis may play an important role in determining how a given tissue responds to an agonist.

We can speculate that cellular Cl^- handling and the endothelial regulation of Cl^- currents may also have pathophysiological significance. The importance of Cl^- currents may be accentuated in regions of localized endothelial dysfunction such as the coronary artery of atherosclerotic patients. Under the diminished influence of NO′, a sudden rise in circulating catecholamines might elicit increased localized Cl^--dependent depolarization and a more sustained contractile response, resulting in ischemia. If this is indeed the case, selective inhibition of Cl^- channel conductance may prove to be a useful strategy for controlling coronary vasospasm.

In summary, we have demonstrated that the endothelium, via NO′, suppresses the degree to which NE-

induced contraction is potentiated by low-Cl^- buffer. In addition, low-Cl^- buffer does not produce contraction in intact rings but consistently does so after endothelial NO′ production is disrupted. These data suggest that NO′ can inhibit the opening of voltage-dependent Ca^{2+} channels by an agonist. This inhibition may be direct (via cGMP inhibition of Ca^{2+} channels) or indirect via prevention of the activation of depolarizing Cl^- currents.

This work was supported through the Children's Health Research Center at the University of Iowa (Iowa City, IA; National Institute of Child Health and Human Development Grant P30-HD-27748) and by a grant from the American Heart Association, Iowa Affiliate.

Address for reprint requests: F. S. Lamb, Dept. of Pediatrics, 5040C RCP, Univ. of Iowa Hospitals, Iowa City, IA 52242.

Received 21 October 1997; accepted in final form 23 March 1998.

REFERENCES

1. **Adams, D. J., J. Barakeh, R. Laskey, and C. Van Breeman.** Ion channels and regulation of intracellular calcium in endothelial cells. *FASEB J.* 3: 2389–2400, 1989.
2. **Casteels, R., K. Kitamura, H. Kuriyama, and H. Suzuki.** The membrane properties of the smooth muscle cells of the rabbit main pulmonary artery. *J. Physiol. (Lond.)* 271: 41–61, 1977.
3. **Chipperfield, A. R., J. P. L. Davis, and A. A. Harper.** An estimate of the permeability ratios of Na, K and Cl in rat femoral arterial smooth muscle measured in vitro (Abstract). *J. Physiol. (Lond.)* 446: 51P, 1992.
4. **Groschner, K., and W. R. Kukovetz.** Voltage-sensitive chloride channels of large conductance in the membrane of pig aortic endothelial cells. *Pflügers Arch.* 421: 209–217, 1992.
5. **Ishikawa, T., J. R. Hume, and K. D. Keef.** Regulation of calcium channels by cAMP and cGMP in vascular smooth muscle cells. *Circ. Res.* 73: 1128–1137, 1993.
6. **Jones, A. W.** Content and fluxes of electrolytes. In: *Handbook of Physiology. The Cardiovascular System. Vascular Smooth Muscle.* Bethesda, MD: Am. Physiol. Soc., 1980, sect. 2, vol. II, chapt. 11, p. 253–299.
7. **Joulou-Schaeffer, G., G. Gray, and I. Fleming.** Loss of vascular responsiveness induced by endotoxin involves the L-arginine pathway. *Am. J. Physiol.* 259 (*Regulatory Integrative Comp. Physiol.* 28): R38–R44, 1990.

7a. **Kaneko, K., and S. Sunano.** Involvement of α-adrenoreceptors in the endothelium-dependent depression of noradrenaline-induced contraction in rat aorta. *Eur. J. Pharmacol.* 240: 195–200, 1993.

8. **Lamb, F. S., and T. J. Barna.** Chloride ion currents contribute functionally to norepinephrine-induced vascular contraction. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H151–H160, 1997.
9. **Laskey, R. E., D. J. Adams, A. Johns, G. M. Rubanyi, and C. Van Breeman.** Membrane potential and Na-K pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine atria. *J. Biol. Chem.* 265: 2613–2619, 1990.
10. **Lorenz, J. N., D. R. Bielefeld, and N. Sperelakis.** Regulation of calcium current in A7r5 vascular smooth muscle cells by cyclic nucleotides. *Am. J. Physiol.* 266 (*Cell Physiol.* 35): C1656-C1663, 1994.
11. **Luckhoff, A., and R. Busse.** Calcium influx into endothelial cells and formation of endothelial-derived relaxing factor is controlled by the membrane potential. *Pflügers Arch.* 416: 305–311, 1990.
12. **Luckhoff, A., U. Pohl, A. Mulsch, and R. Busse.** Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. *Br. J. Pharmacol.* 95: 189–196, 1988.
13. **Nilius, B., M. Oike, I. Zahradnik, and G. Droogmans.** Activation of a Cl current by hypotonic volume increase in human endothelial cells. *J. Gen. Physiol.* 103: 787–805, 1994.
14. **Quignard, J. F., J. M. Frapier, M. C. Harricane, B. Albat, J. Nargeot, and S. Richard.** Voltage-gated calcium channel currents in human coronary myocytes. Regulation by cyclic GMP and nitric oxide. *J. Clin. Invest.* 99: 185–193, 1997.
15. **Rees, D. D., R. M. J. Palmer, and S. Moncada.** Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. USA* 86: 3375–3378, 1989.
16. **Salomone, S., N. Morel, and T. Godfraind.** Effects of 8-bromo cyclic GMP and verapamil on depolarization-evoked calcium signal and contraction in rat aorta. *Br. J. Pharmacol.* 114: 1731–1737, 1995.
17. **Smith, J. M., and A. W. Jones.** Calcium-dependent fluxes of potassium-42 and chloride-36 during norepinephrine activation of rat aorta. *Circ. Res.* 56: 507–516, 1985.
18. **Vanhoutte, P. M.** Endothelial-derived hyperpolarizing factor. In: *Endothelial Cell Research Series* (1st ed.), edited by G. M. Rubanyi. Amsterdam: Harwood Academic, 1996, p. 338.
19. **Watanabe, M., K. Yumoto, and R. Ochi.** Indirect activation by internal calcium of chloride channels in endothelial cells. *Jpn. J. Physiol.* 44: S233–S236, 1994.
20. **Wiklund, N. P., M. G. Persson, L. E. Gustafsson, S. Moncada, and P. Hedquist.** Modulatory role of endogenous nitric oxide in pulmonary circulation in vivo. *Eur. J. Pharmacol.* 185: 123–124, 1990.
21. **Yumoto, K., H. Yamaguchi, and R. Ochi.** Depression of ATP-induced calcium signalling by high K and low Cl media in human aortic endothelial cells. *Jpn. J. Physiol.* 45: 111–122, 1995.

(10) Related Proceedings Appendix.

There have been no decisions rendered by a court or the Board in the appeal of Application Serial No. 09/930,105.